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PURIFICATION OF SEMLIKI FOREST VIRUS RNA-DEPENDENT

RNA POLYMERASE

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Thesis presented for the degree of Doctor of Philosophy,  
Department of Biological Sciences, University of Warwick,  
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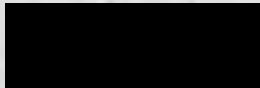
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Finally, my thanks to my mother and father who made all this possible.

### Declaration

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree. The work of which it is a record has been done by myself and any contributions by others have been specifically acknowledged. In particular, the section on the polypeptide composition of the replicase has drawn heavily on the unpublished observations of Dr. C. Clegg.



Abbreviations

A	Adenylate
AMD	Actinomycin D
ATP	Adenosine 5'-triphosphate
BHK	Baby hamster kidney
C	Cytidylate
CEC	Chick embryo cells
Ci	Curie ( $3.7 \times 10^{10}$ disintegrations per sec)
Ct	Count
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOC	Sodium deoxycholate
DS500	Dextran sulphate 500
dT	Deoxythymidylate
DTT	Dithiothreitol (Clelands reagent)
EDTA	Ethylenediamine tetra-acetic acid (Sequestric acid)
EEE	Eastern Encephalitis virus
EMC	Encephalomyocarditis virus
FMDV	Foot and Mouth disease virus
GTP	Guanosine 5'-triphosphate
HA	Haemagglutinin
LDH	Lactate dehydrogenase
M	Molarity
m.o.i.	Multiplicity of infection
MW	Molecular weight
N	Normality
NA	Neuraminidase
NAD+	Nicotinamide adenine dinucleotide

NADP+	Nicotinamide adenine dinucleotide phosphate
NDS	Naphthalene 1,5-disulphonic acid
PBS	Phosphate buffered saline
p.f.u.	Plaque forming unit
P.I.	Post-infection
PVS	Polyvinyl sulphate
RF	Replicative form
RI	Replicative intermediate
RK	Rabbit kidney
RNA	Ribonucleic acid
RNase	Ribonuclease
S	Sedimentation coefficient
SSC	Standard saline citrate
SDS	Sodium dodecyl sulphate
SFV	Semliki Forest virus
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
UTP	Uridine 5'-triphosphate
VEE	Venezuelan Encephalitis virus
VSV	Vesicular Stomatitis virus
v/v	volume for volume
WEE	Western Encephalitis virus
w/v	weight for volume
w/w	weight for weight

### SUMMARY

The RNA-dependent RNA polymerase (RNA replicase) specified by Semliki Forest Virus has been studied. The enzyme, rapidly synthesized early in infection, was present at the greatest activity by 4hr post-infection and, at this time, the activity of the enzyme became independent of further protein synthesis.

Replicase activity was found in the cytoplasm of the infected cell and, after removal of nuclei, the replication complex was characterized by isopycnic sucrose gradient centrifugation. A membranous band at 35% (w/w) sucrose contained replicase activity and was the site of RNA synthesis. At 1 $\frac{1}{2}$ hr post-infection the replicase was associated with input virion RNA but not input virion proteins.

Replicase present in the post-nuclear supernatant was competent to synthesize all forms of virus-specific RNA found in the infected cell. However, on further purification there was a loss of the production of discrete single-stranded RNA in vitro. This loss was probably caused by endogenous ribonuclease activity and disruption of the replication complex.

Recovery of the replicase rich band from isopycnic gradients allowed it to be analysed on linear sucrose gradients. The replication complex had an S-value of 250 which, on non-ionic detergent treatment, was converted to a 25S structure. The replicase activity of this 25S complex was specifically bound to an oligo(dT)-cellulose column bearing attached 42S virion RNA. Analysis of the polypeptides eluted off

in the fractions having replicase activity showed that there were only 3 labelled bands visible on polyacrylamide gels. Two of these comigrated with two non-structural virus polypeptides identified in infected cells. The third polypeptide of the purified replicase preparation (MW 40,000 daltons) is probably a host protein. Material from mock-infected cells purified exactly as the replicase contained only the 40,000 dalton protein.

It is therefore concluded that SFV replicase contains 2 virus-specific polypeptides which have MWs of 90,000 and 63,000 daltons.

## INTRODUCTION

### 1. Biological aspects of Semliki Forest Virus (SFV)

#### a) Classification.

In 1942 virus was isolated from a pool of 130 female Aedes abnormalis mosquitoes captured in Theobald, Bwanba, Uganda (Smithburn and Haddow, 1944). This virus, named Semliki Forest Virus, was originally classified as a group A arbovirus (Casals and Brown, 1954) together with Sindbis virus, Eastern (EEE), Western (WEE) and Venezuelan Encephalitis (VEE) viruses on the basis of immunological data. Since there were viruses classified with the arboviruses but which are not arthropod-borne (e.g. lactic dehydrogenase virus of mice (Melnick, 1969)) and arthropod transmission has been found for structural groups other than the arboviruses (e.g. members of the reo-group of viruses) it was suggested that the name togavirus be used to cover the A and B groups of the arboviruses (Andrewes, 1970). Thus all that the name arbovirus implies is that the virus has a life cycle encompassing both arthropods and vertebrates. SFV and Sindbis virus (with other group A arboviruses) are now classified in the genus Alphavirus, family Togaviridae (Wildy, 1971; the cryptogram is given as R/1: 4/6: S/S: V,I/Di). The group B arboviruses are placed in the genus Flavivirus.

The togaviruses are grouped with the picornaviruses as Class IV viruses according to the Baltimore scheme (Baltimore, 1971).

#### b) Distribution and Ecology.

The distribution of SFV is known only on the basis of three isolates from Aedes or Eretmapodites mosquitoes in Uganda, Mozambique and Cameroon. Occurrence in Malaya and

North Borneo has been suggested on the basis of serological studies (cited in Andrewes and Pereira, 1972). The natural hosts and vectors are unknown (Andrewes and Pereira, 1972) although antibodies have been found in the sera of man and wild primates in Uganda (Smithburn, Mahaffy and Haddow, 1944). Collins (1963) found that anopheline mosquitoes could transmit the infection experimentally. Sindbis virus, antibodies to which have been found in man, is reported to have caused illness in five patients in Uganda (cited in Horsfall and Tamm, 1965).

c) Host Range.

In the laboratory SFV grows well in the brains of newborn mice (Cheng, 1961). Henderson and Taylor (1960) found that various alphaviruses (SFV, Sindbis) grew in chick embryo, Pekin duck kidney or embryo, and rhesus monkey kidney cells whereas flaviviruses (West Nile, Japanese<sup>B</sup>) did not grow in chick embryo or rhesus monkey kidney cells (except for Ilheus virus). Unclassified arboviruses (previously group C), Oriboca and Murutucu only grew in rhesus monkey kidney cells. High titre SFV can be grown in suspension culture chick embryo cells (Zwartouw and Algar, 1968). Bergold and Mazzali (1968) described the titration of 52 viruses, 20 virus strains (which were then classified as arboviruses) in BHK, Vero (Green monkey), rhesus monkey kidney and rabbit kidney cells. Hep-2 cell cultures have been used to study the morphological development of SFV (Erlandson et al. 1967). SFV grows with similar kinetics in cultured Aedes albopictus cells as in pig kidney, vero and chick embryo cells (Davey, Dennett and Dalgarno, 1973).

The virulence of various original and derived strains of SFV has been studied by administration to mice, Guinea-pigs and rabbits (Bradish, Allner and Maber, 1971). From this work



it was concluded that there was no correlation between avirulence and inefficiency of infection or replication. Similarly there is no correlation between virulence and rates of inactivation, electrophoretic and chromatographic distribution patterns of the virus (Fleming, 1973). The use of immunosuppressants may help elucidate the nature of expression of virulence (Allner et al., 1974).

Persistent infection by SFV has been reported in Aedes albopictus cells (Davey and Dalgarno, 1974) and by Sindbis virus in *Drosophila* cell cultures (Bras-Herren, 1975), in mouse cells (Ingnot, Albin and Chudzio, 1973), in BHK-21 cell cultures (Schwöbel and Ahl, 1972).

Thus, the togaviruses are widely distributed and are able to infect a variety of cell cultures in experimental conditions, causing many different cytopathic and morphological changes. The most extensively studied are SFV and Sindbis virus which can be taken as representative of, at least, alphaviruses (whether the flaviviruses have a similar multiplication cycle is not known).

## 2. The structure of the virion.

### a) Chemical composition

The chemical composition of SFV grown in BHK cells has been estimated to be 6.3% RNA, 12.2% nucleocapsid protein, 44.4% envelope polypeptides, 30.8% lipid and 6.3% protein bound carbohydrate (Laine, Söderlund and Renkonen, 1973). The lipid composition has been reported as 26.5% sphingomyelin, 34% lecithin, 22.8% phosphatidylethanolamine, 15% phosphatidylinositol and 0.8% phosphatidylserine (Friedman and Pastan, 1969). The monosaccharides of the protein bound carbohydrate include N-acetyl-glucosamine, N-acetyl-galactosamine, galactose, glucose, fucose and sialic acid (Laine,

Söderlund and Renkonen, 1973) together with mannose and a glucose containing glycolipid (Oram et al., 1971). Those of Sindbis include glucosamine, mannose, galactose, fucose and sialic acid. The sialic acid content depends on whether the virus is grown in chick or hamster cells (Strauss, Burge and Darnell, 1970; Burge and Strauss, 1970).

#### b) Architecture.

When observed in the electron microscope SFV is seen as a spherical particle 60-80nm in diameter. A membrane, covered with 6-11nm projections, encloses a nucleocapsid 30-40nm in diameter (Acheson and Tamm, 1967; Simpson and Hauser, 1968). X-ray diffraction studies reveal that four non-overlapping zones can be distinguished in the virus particles: RNA, core protein, lipid and envelope protein (Harrison et al., 1971). The RNA, associated with core protein (Burge and Strauss, 1970) comprises the nucleocapsid and is surrounded by a lipoprotein envelope (Osterrieth and Carlberg-Bacq, 1966) revealed by X-ray diffraction to be in bilayer form (Harrison et al., 1971).

The components of the virus particle will be considered in turn.

#### (1) RNA

The RNA extracted from SFV particles has a sedimentation coefficient (S value) on sucrose gradients of 42 (Friedman, Levy and Carter, 1966; Friedman and Berezesky, 1967; Sonnabend, Martin and Mécs, 1967; Cartwright and Burke, 1970). The molecular weight of this RNA has been determined by polyacrylamide gel electrophoresis to be  $4 \pm 0.08 \times 10^6$  daltons (Levin and Friedman, 1971; Martin and Burke, 1974). Sindbis virion RNA has a similar S value and molecular weight (Dobos and Faulkner, 1970; Simons and Strauss, 1972a).

It was reported that the virion RNA of WEE (40S) could be converted to RNA with a coefficient of 26S thermally, by dialysis against water, by 8M urea or by treatment with 50% DMSO. It was further claimed that the 26S differed from 40S only in secondary structure and was infectious (Sreevalsan et al., 1968). For Sindbis (Dobos and Faulkner, 1970; Boulton and Westaway, 1972) and SFV (Cartwright and Burke, 1970) it was reported that, on denaturation of virion RNA, a fragment the same size as intracellular viral 26S RNA (see later) was formed. On the basis of this work and the observation that the base compositions of the 26S and 42S were similar (Friedman and Berezesky, 1967) it was suggested that 42S RNA consists of two 26S species hydrogen bonded together. This is now known to be incorrect. Fragmentation of the genome RNA occurs because of the introduction of hidden breaks during extraction (Arif and Faulkner, 1972).

The base composition of the virion RNA is 25.9% cytosine, 25.5% guanine, 29.1% adenine and 19.5% uracil (Kääriäinen and Gomatos, 1969) similar values being reported by Sonabend et al (1967) and Pfefferkorn and Hunter, 1963b).

Sindbis virion RNA contains adenylate rich regions (poly (A)) (Johnston and Bose, 1972a, b). The RNA showed heterogeneity in poly (A) content; 80 to 90% of the RNA molecules isolated contained a poly (A) sequence 60-80 nucleotides in length, the remainder contained a larger poly (A) species of 150-250 nucleotides. The two classes of molecules were equally infectious (Eaton and Faulkner, 1972). The 3'-terminal residue of SFV and Sindbis RNA was shown to be uridine as determined by borohydride labelling. After a limited snake venome exonuclease digest the poly (A) sequence of Sindbis virion RNA bound to Millipore membranes was concluded to be at least

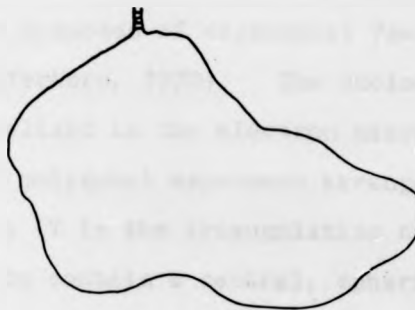
6% in from the 3'-terminus (Donaghue and Faulkner, 1973). Hybridization of poly (dT) with Sindbis virion RNA, and examination of the hybrid using an electron microscope showed that a large proportion of the molecules were present as structures interpreted as having the conformation shown in Figure 1. This indicates that the poly (A) is terminal on the RNA (Hsu, Kung and Davidson, 1973). These authors also found that, depending on the conditions, some of the RNA molecules (both with and without poly (dT) were present as a circular form with a duplex "handle" (Figure 1). Thus Sindbis RNA has cohesive ends and may exist in the virion as a circular molecule. Interestingly, circular molecules have also been found amongst purified replicative form RNA of the picornavirus EMC (Agol et al. 1972).

The 42S virion RNA of SFV has been calculated to contain 100-110 residues of poly (A) (Clegg and Kennedy, 1974a). The RNA does not have a poly (C) tract as do some picornaviruses, e.g. EMC, Mengo viruses (Porter, Carey and Fellner, 1974; Brown et al. 1974). There is evidence that the 5'-end is "capped" (S.I.T. Kennedy, personal communication) as has been reported for several virus RNAs, eg. reo, VSV (Shatkin et al. 1975) but interestingly, not for the picornaviruses, which belong to the same group as SFV (Baltimore, 1971).

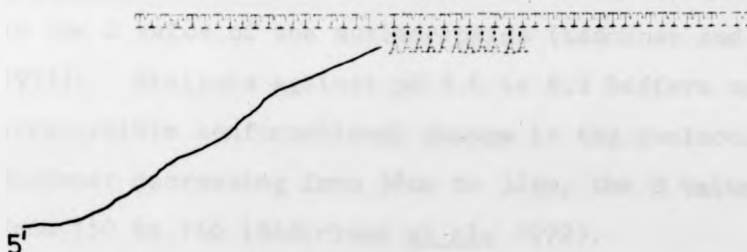
#### (ii) The nucleocapsid

Nucleocapsids contain 38% RNA and only one type of polypeptide chain, the core protein, MW 32,000 daltons (Acheson and Tamm, 1970b; Kääriäinen, Simons and von Bonsdorff, 1969). Based on the MW of the core protein and the % protein in nucleocapsids, Laine, Söderlund and Renkonen <sup>(1973)</sup> calculated that there is one RNA chain surrounded by 230 molecules of core protein.

Figure 1. Schematic representation of Sindbis virus RNA seen by Hsu, Kung and Davidson (1973). (Not drawn to scale).



a. Circular molecules with a duplex handle of  $250 \pm 50$  nucleotide pairs.



b. Y-shaped structures observed on hybridization of poly(dT) with Sindbis RNA showing that the poly(A) sequence is terminal.



c. Circular molecules observed with dT attached to the handle suggesting that the poly(A) sequence is not all, or only partially involved in base pairing of the cohesive ends.

The nucleocapsid, liberated by treatment of the virus with detergent, has been estimated to be 30nm in diameter with an S value of 145 (Acheson and Tamm, 1970a), and 38nm in diameter with an S value of 150 (Käärinen and Söderlund, 1971). It is composed of capsomeres 7nm in diameter (Brown, Waite and Pfefferkorn, 1972). The nucleocapsid of Sindbis virus was visualized in the electron microscope as consisting of 32 "hollow" polygonal capsomers arranged within a T=3 surface lattice (T is the triangulation number). The core was also seen to contain a central, spherical structure 12 to 16nm in diameter (Horzinek and Mussgay, 1969).

Treatment of nucleocapsids with pancreatic ribonuclease causes a digestion of the contained RNA leading to a decrease in the S value of the nucleocapsids (Käärinen and Söderlund, 1971). Dialysis against pH 5.6 to 6.2 buffers causes an irreversible conformational change in the nucleocapsid; the diameter decreasing from 38nm to 32nm, the S value increasing from 150 to 166 (Söderlund *et al.*, 1972).

The early reports suggesting that there are two proteins in the nucleocapsid (Friedman, 1968c) have been shown to be incorrect. The small amounts of one protein present on polyacrylamide gels was probably a dimer of the core protein (Acheson and Tamm, 1970b). In the presence of a sulphhydryl reductant only one protein species is found in the nucleocapsid (Hay, Skehel and Burke, 1968). Amino acid analysis of the core protein revealed that it is rich in hydrophilic amino acids, particularly lysine and glutamate. The N-terminal amino acid of the core protein is lysine (Kennedy and Burke, 1972). These authors also obtained tryptic peptide maps of the core and one structural protein, the envelope protein.

Thus the icosahedral nucleocapsid consists of RNA partially protected by core protein molecules arranged in capsomers (if there are 230 molecules of core protein and 32 capsomeres, then there are approximately 7 molecules of core per capsomer).

(iii) The lipid envelope

The nucleocapsid is surrounded by a lipid bilayer. If the envelope glycoproteins of SFV are removed by bromelain digestion a lipid containing particle is left. This particle is susceptible to phospholipase C, producing a core indistinguishable, by sedimentation analysis, from nucleocapsids found in infected cells (Kennedy, 1974).

Renkonen et al (1971) found that the lipid composition of SFV resembled, but was not identical, to that of the host cell plasma membrane whilst David (1971) found that the lipids of Sindbis were very similar whether the virus was grown in chick or hamster cells. This finding led David to postulate that the lipid composition of the viral envelope reflects the lipid affinities of viral envelope polypeptides. Friedman and Pastan (1969) suggested that the phospholipids stabilize the virus.

(iv) The envelope proteins

Initially it was thought that the envelope protein of SFV consisted of a single polypeptide (Friedman, 1968a; Hay, Skehel and Burke, 1968; Kääriäinen, Simons and von Bonsdorff, 1969; Acheson and Tamm, 1970b; Kennedy and Burke, 1972). Similarly, Sindbis virus was reported to have only one envelope protein (Strauss et al, 1968; Strauss, Burge and Darnell, 1968). Using a discontinuous SDS gel system the 53,000 dalton MW envelope protein of Sindbis was resolved into two glycoproteins ( $E_1$  53,000 daltons,  $E_2$  45,000 daltons). These were shown to

be discrete glycoproteins as  $E_2$  contains more arginine and less methionine than  $E_1$ . Incorporation of labelled precursors into the 3 resolved viral proteins indicated that they were present in a molar ratio of 1 : 1 : 1 (Schlesinger, Schlesinger and Burge, 1972). The envelope protein of SFV was similarly resolved into two glycoproteins (Simons, Keranen and Kääriäinen, 1973; Kennedy, 1974). Using the technique of SDS-hydroxylapatite chromatography Garoff, Simons and Renkonen (1974) isolated a third glycoprotein ( $E_3$ ) from the SFV membrane. They suggested that it is a minor component and is not detectable by protein staining of SDS gel because 82% of the protein MW is carbohydrate. The MWs of  $E_1$  and  $E_2$  were estimated to be 49,000 and 53,000 daltons respectively (by SDS-polyacrylamide gel electrophoresis) and 10,000 daltons for  $E_3$  (by chemical composition).

The glycoproteins isolated from vesicular stomatitis virus (VSV) and Sindbis virus grown in the same host differ principally in the number of sialic acid residues per glycopeptide (Burge and Huang, 1970). Sialyl transferase isolated from uninfected cells is capable of transferring sialic acid to acceptor prepared from Sindbis membrane glycoprotein indicating that some of the carbohydrate of the glycoprotein is derived by host modification.

The spike-like projections observed on the surface of SFV (Osterrieth and Calberq-Bacq, 1966) have been visualized in the electron microscope as 4nm subunits with a centre to centre spacing of 6nm (Brown, Waite and Pfefferkorn, 1972) and are destroyed by caseinase C leading to a loss of haemagglutinating activity (HA), but little loss of infectivity (Osterrieth, 1966). The nucleocapsid is not agglutinated by the phytohaemagglutinin concanavalin A which binds specific



sugar residues and is able to agglutinate purified SFV and its envelope glycoproteins (Oram et al., 1971). Thus the spikes contain carbohydrate residues and are composed of the envelope glycoproteins.

When the virus particle is treated with thermolysin or subtilisin, the spikes, corresponding to  $E_1$  and  $E_2$ , are cleaved off leaving hydrophobic peptide segments (MW approximately 5,000 daltons) in the membrane (Utermann and Simons, 1974). The hydrophobic segments presumably anchor the glycoprotein in the lipid bilayer and are protected against proteolysis by lipid in the membrane. Further, the spike glycoproteins extend through the viral membrane and are in close contact with the nucleocapsid. This was shown using the crosslinking reagent dimethylsuberimide (DMS) which crosslinks protein amino groups up to 1.1 nm apart. DMS links the membrane protein to the nucleocapsid, so the protein must pass through the 4nm lipid bilayer (Garoff and Simons, 1974). Electron micrographs of negatively stained Sindbis virus particles show that the glycoproteins project 8nm outwards and are organised with trimer clustering in a  $T=4$  icosahedral surface lattice. The inner polar groups of the lipid bilayer connect the nucleocapsid and lie at a radius of 21nm. The outer polar groups are at a radius of about 26nm (von Bonsdorff and Harrison, 1975).  $E_1$  is less accessible to iodination than  $E_2$  using lactoperoxidase which labels only surface proteins (Sefton, Wickus and Burge, 1973) so  $E_2$  may protrude further than  $E_1$ .

(v) Enzymic and biological activities of the virus particle

There are several measurable activities associated with SFV eg. infectivity, HA activity and antigenic properties. Digestion of the envelope glycoproteins by bromelain destroys

infectivity, HA activity and surface antigenicity, whereas the removal of sialic acid by neuraminidase has no effect on these properties (Kennedy, 1974). The protein portion of the glycoprotein is apparently involved in biological activities, the carbohydrate moiety being involved in hydrophilic interactions.

Nonidet P-40, deoxycholate and Tween 80/ether all destroy virus infectivity whilst preserving complement fixing, HA and neutralising antibody (NA) blocking activities. The HA and NA-blocking activities are associated with released envelope, the complement fixing antigens are detected in the nucleocapsid and envelope (Appleyard, Oram and Stanley, 1970 ; Bose and Sagik, 1970a).

Protein kinase (80 times lower than the activity found in VSV) and phosphoprotein phosphatase are associated with virions, the nucleocapsids being the repository of the greater specific activity of both enzymes (Tan and Sokol, 1974).  $E_1$  and  $E_2$  are phosphorylated, containing 0.03 to 0.1 mole of phosphate per mole of protein. The phosphorylated amino acids are serine and threonine (Waite et al., 1974).

### 3. The multiplication cycle

#### a) Infection

The adsorption, penetration and subsequent transport of viruses and virus macromolecules is not well understood (see Dimmock, 1975). Whilst virion RNA from SFV is infectious (Sonnenbend, Martin and Mecs, 1966; Mecs et al., 1967a; Friedman, Levy and Carter, 1967), the nucleocapsids are not, being unable to attach to host cells (Bose and Sagik, 1970b). Thus the virus envelope proteins are necessary for adsorption to host cells. Adsorption is very rapid, 40 to 60% of particles of Sindbis ultimately attached are bound within one minute

(Purifoy and Sagik, 1968). The adsorption of Sindbis to glutaraldehyde fixed cells at 37°C, visualized in the electron microscope, indicated that there are about  $10^5$  receptors per chick cell at a concentration of 20-160 per  $\mu\text{m}^2$  of cell surface. Virus particles adsorbed to unfixed cells at 4°C cluster into aggregates indicating that there is movement of virus or receptors (Birdwell and Strauss, 1974). Mannosamine inhibits an early stage in the replication of SFV, glucosamine a late stage, and using those sugars with 5 strains of virus differing in virulence Fleming (1973) suggested that the strains have different attachment and maturation sites. The binding of Sindbis to liposomes from sheep erythrocytes is dependent on pH. In the absence of cholesterol or phosphatidylethanolamine the binding is reduced (Mooney et al., 1975).

#### b) Maturation

Budding of virus occurs in patches over the cell surface, and in some cells occurs primarily at the peripheral surface through long extensions of the cell, 7  $\mu\text{m}$  long, over 50nm in diameter (Birdwell, Strauss and Strauss, 1973). |

The nucleocapsids may migrate together so giving rise to the "patch" phenomena. Since the envelope proteins are inserted in the plasma membrane independently of the nucleocapsids, for mature virus to be produced, the nucleocapsids must migrate to those areas of the cell containing the envelope proteins. The alternative hypothesis, that the incorporation into the cell membrane of the glycoprotein is under the control of the nucleocapsid has not been ruled out (Pedersen and Sagik, 1973). Pertinent to this is that nucleocapsid assembly occurs more rapidly than insertion of envelope proteins into the cell membrane (Schaele and

Pfefferkorn, 1969a).

Garoff and Simons (1974) have proposed a model for the assembly of virus particles which envisages  $E_1$  and/or  $E_2$  spanning the plasma membrane. A nucleocapsid would bind to an exposed spike glycoprotein and then act as a nucleation site for additional glycoproteins moving into the growing patch in the plasma membrane by lateral diffusion. The release of the mature virus particle occurs when all the binding sites on the nucleocapsid are filled.

Infected cells, 3 to 5hr after infection, are agglutinated to a greater extent by concanavalin than uninfected cells. This suggests that the insertion of virus glycoproteins into the membrane is accompanied by a rearrangement of receptor sites in the cell surface (Birdwell and Strauss, 1973). Electron micrographs of freeze etched budding virus have been interpreted as showing that the fusion of the cell membrane at the base of the budding virion is a two step process; the inner surface fusing into a sphere before the outer one (Brown, Waite and Pfefferkorn, 1972). Release of virus is inhibited at  $4^{\circ}\text{C}$  (Brown, Waite and Pfefferkorn, 1972) and is dependent on continuing protein synthesis (Scheele and Pfefferkorn, 1969a) arguing that it is a dynamic rather than a passive process.

#### c) Association with membranes

The replication of viruses is dependent on the host cells protein synthesizing system and involves a rearrangement of the membrane system of the host cell. SFV and Sindbis virus will replicate in enucleate cells indicating that the presence of a nucleus is not obligatory for successful virus infection and multiplication (Follett, Pringle and Pennington, 1975 ; Kos, Osborne and Goldsby, 1975). Japanese encephalitis virus, a

flavivirus, does not replicate or produce virus specific antigen in chick embryo cells enucleated 2hr post-infection indicating that this togavirus requires the cell nucleus early in infection (Kos, Osborne and Goldsby, 1975).

Viral multiplication is intimately involved with membranous structures, both pre-existing and viral induced. Friedman and Grimley (1967) described the accumulation of viral RNA in large cytoplasmic bodies containing nucleocapsids. RNA synthesis occurs on so-called type 1 cytopathic vacuoles (CPV-1) characterized by membrane buds attached to their inner surface. Type 2 cytopathic vacuoles (CPV-2), numerous late in infection, contain many nucleocapsids located on their exterior (Grimley, Berezesky and Friedman, 1968). The association of the replicative intermediate (a multistranded, partially ribonuclease resistant structure (Franklin, 1966) and the RNA replicase with membranes suggests that RNA is formed at CPV-1 structures (Friedman et al., 1972). Sindbis virus RNA and coat protein synthesis is similarly located in the cytoplasmic reticulum (Ben-Ishai, Goldblum and Becker, 1968).

CPV-1 are formed from pre-existing host cell macromolecules and appear 3 to 6hr post-infection, preceding the accumulation of nucleocapsids. The formation of CPV-1 is insensitive to Actinomycin D, but sensitive to guanidine and cycloheximide (Grimley et al., 1972). The synthesis of CPV-2 is much more sensitive to cycloheximide than CPV-1, the former, which together with nucleocapsids, are not found if the drug is present 4hr post-infection (Friedman and Grimley, 1968).

As well as RNA and its synthesizing activity, envelope and core proteins are associated with plasma membrane fractions isolated from chick embryo cells infected with Sindbis virus.

#### d) Viral RNA forms

By sucrose density gradient sedimentation 3 species of RNA can be resolved: 42S and 26S ribonuclease-sensitive forms and a 20S ribonuclease-resistant form (Friedman, Levy and Carter, 1966; Sonabend, Martin and Mecs, 1967). If cells are infected with virus having radiolabelled virion RNA, the label is detectable in a replicative intermediate (RI) having similar properties to the RIs described in cells infected with RNA bacteriophages (Friedman, 1968b). Treatment of RI with ribonuclease yields a 20S ribonuclease-resistant core, the replicative form (RF). By polyacrylamide gel electrophoresis Levin and Friedman (1971) calculated the molecular weights of the RNA forms to be for 42S RNA,  $4 \times 10^6$  daltons; for 26S RNA,  $1.8 \times 10^6$  daltons. They also resolved two more ribonuclease-sensitive forms of RNA whose S values and molecular weights were reported as: 38S RNA,  $3.1 \times 10^6$  daltons; 33S RNA,  $2.4 \times 10^6$  daltons. These values were confirmed by Martin and Burke (1974). The 33S RNA contains 90% of the base sequences of the 26S RNA as shown by hybridization-competition experiments (Simmons and Strauss, 1972a) and has identical characteristic oligonucleotides to the 26S RNA after a complete  $T_1$  ribonuclease digest (S.I.T. Kennedy, personal communication). The 33S RNA is therefore thought to be a conformational variant of the 26S RNA. Similarly the 38S RNA may be a conformational variant of the 42S RNA (S.I.T. Kennedy, personal communication).

After ribonuclease treatment of viral RNA extracted from infected cells, the RF core has been resolved into more than one species (Levin and Friedman, 1971; Stollar, Shenk and Stollar, 1972; Simmons and Strauss, 1972b; Martin and Burke, 1974). Their molecular weights have been estimated to be

$8.8 \times 10^6$ ,  $5.6 \times 10^6$  and  $2.9 \times 10^6$  daltons, RFI core, RFII core and RFIII core, respectively (Simons and Strauss, 1972b). RFII core and RFIII core exist in a one-to-one molar ratio throughout the infection cycle and apparently arise from breakdown of RFI. The RFI is probably the core of the RI which is producing single stranded RNA. Infectious RNA can be isolated from the RI and RF core (Segal and Sreevalsan, 1974).

RFI, II and III can be isolated from infected cells without ribonuclease treatment. RFI consists of a duplex of two 42S RNA molecules, one of positive polarity and the other of negative, and constitutes more than 80% of the total replicative form (Bruton and Kennedy, 1975). The RI consists of an intact 42S RNA strand of negative polarity, this being the only RNA of negative polarity which has been isolated from infected cells. If, as Simons and Strauss (1972b) and Segal and Sreevalsan (1974) have suggested, the synthesis of 42S and 26S RNAs occurs on two distinct replication complexes, then both must use 42S negative strand as template.

The single-stranded RNA species all have positive polarity (Martin and Burke, 1974) and possess poly (A) tracts (Clegg and Kennedy, 1974a). In addition, the 20S double-stranded RNA, the 42S positive strand of RFI and 30% of the RI molecules contain poly (A) (Clegg and Kennedy, 1974a; Bruton and Kennedy, 1975). Polyribosomal-associated RNA contains poly (A) (Eaton, Donaghue and Faulkner, 1972). The major species of RNA found on the polyribosomes is 26S, (Kennedy, 1972). Small amounts of 42S and 33S RNA are also found (Kennedy, 1972; Mowshowitz, 1973; Söderlund, Glanville and Kääriäinen, 1974; Wengler and Wengler, 1974). Rosemond and Sreevalsan (1973) found another single-stranded species of RNA, 15-18S,

associated with ribosomes, but this is probably RNA associated with defective virus particles. About 50% of the RNA, 2 to 4hr after infection, is associated with polyribosomes (Wengler and Wengler, 1974), the rest is either associated with the nucleocapsids or with a ribonucleoprotein (RNP) complex sedimenting at 55 to 65S on sucrose gradients (Friedman and Berezesky, 1967; Simons and Strauss, 1974a; Wengler and Wengler, 1974). 42S RNA isolated from these different structures is identical as regards infectivity, sedimentation in the presence of formamide and translation in vitro (Wengler and Wengler, 1975a). The 55S RNP complex may be an intermediate in the formation of nucleocapsids.

Thus, the 42S and 26S RNAs are the messengers (see later) in infected cells; the 26S RNA representing 90% of the viral mRNA, the 42S constituting the remaining 10% (Simons and Strauss, 1972a).

Some of the double-stranded RNAs are precursors to single-stranded RNAs (Kääriäinen and Gomatos, 1969; Scholtissek, Kalaza and Rott, 1972; Michel and Gomatos, 1973). The double-stranded RNA is the first newly synthesized RNA (Cartwright and Burke, 1970) and is membrane bound (Kääriäinen and Gomatos, 1969). The 26S RNA is detectable 30 min after the RF and RI (which are detectable 1hr after infection). Maximal synthesis of 42S viral RNA occurs 4 to 5½hr after infection, when there is maximal accumulation of newly synthesized virions.

Negative strands of 42S RNA, in a complex, are detectable 1½hr after infection and increase rapidly until 2½hr, when the rate of synthesis decreases, being undetectable 4hr after infection (Bruton and Kennedy, 1975). The rate of synthesis of positive strands increases up to 2½hr after infection when the rate remains constant until 6hr after infection.



e) Viral specific proteins

Besides the structural proteins of the virion several other new polypeptides can be detected in cells infected with alphaviruses. For SFV 4 non-structural polypeptides were first described (Hay, Skehel and Burke, 1968), for Sindbis 12 to 16 polypeptides were detected in chick and hamster cells (Strauss, Burge and Darnell, 1969). Two of the non-virion polypeptides (NVPs) are glycosylated, NVP-97 (MW, 97000 daltons) and NVP-63 (MW, 63,000 daltons) whilst one is not, NVP-78 (MW, 78,000 daltons) (Morser, Kennedy and Burke, 1973). NVP-97 is cleaved to give  $E_1$  and NVP-63 which is itself cleaved to give  $E_2$  and  $E_3$  (Simons, Keranen and Kääriäinen, 1973). A similar pattern of cleavage of Sindbis virus specified polypeptides has been demonstrated (Schlesinger and Schlesinger, 1972; Snyder and Sreevalsan, 1974). It may be that a larger precursor molecule exists; in cells treated with amino acid analogues and inhibitors of proteolytic enzymes larger molecules (of sufficient size) can be detected (Pfefferkorn and Boyle, 1972; Morser, Kennedy and Burke, 1974). Certain temperature-sensitive mutants, on infection at the non-permissive temperature give rise to large MW polypeptides containing the tryptic peptides present in core and envelope proteins (Schlesinger and Schlesinger, 1973). The uncleaved precursor (NVP-63) to the envelope proteins is found associated with the plasma membrane fraction of infected cells (Jones, Waite and Bose, 1974).

The structural proteins of the virus are coded for by the 26S RNA found in infected cells. When this message is used to direct cell free protein synthesis the core and envelope proteins are formed (Clegg and Kennedy, 1974b) or the core and a precursor to the envelope proteins (Simons and Strauss, 1974b) or only discrete core protein (Cancedda and Schlesinger, 1974;

Cancedda, Swanson and Schlesinger, 1974; Wengler, Beato and Hackemack, 1974). If, after initiation of protein synthesis on the 26S messenger RNA in vitro, elongation is inhibited by diphtheria toxin and NAD only one dipeptide, methionyl-asparagine, can be detected (Clegg and Kennedy, 1975b). The restoration to isotonicity of BHK cells infected with SFV, which have been exposed to medium containing a high concentration of NaCl, induces the synchronous initiation of protein synthesis. Pulse labelling and pulse-chase experiments with this system has allowed the order in which the structural proteins are synthesized to be determined as core, E<sub>3</sub>, E<sub>2</sub> and finally E<sub>1</sub>, in the 5' to 3' direction (Clegg, 1975; Clegg and Kennedy, 1975b). Thus there is a single initiation site on the 26S RNA, and since the N-terminal amino acid of the core is lysine (Kennedy and Burke, 1972) there must be a lead-in sequence of amino acids before translation of the core protein begins. The MW of the 4 structural proteins together is about 141,000; the coding capacity of the 26S RNA (MW  $1.8 \times 10^6$  daltons, Martin and Burke, 1974)) is about 180,000, thus there is some part of the 26S RNA which is either not translated or does not code for structural proteins. Further, since the 26S RNA represents a unique one-third of the 42S RNA (Simmons and Strauss, 1972a) and is located at the 3' end of the 42S RNA (S.I.T. Kennedy, personal communication), there must be at least two initiation sites on the 42S RNA; one at or near the 5' terminus, the other corresponding to the initiation site on the 26S RNA.

The translation of 42S virion RNA in vitro gives rise to non-structural polypeptides which have not been correlated with those found in infected cells (Simmons and Strauss, 1972b), and core protein (Cancedda and Schlesinger, 1974; Cancedda,

Swanson and Schlesinger, 1974), and peptides characteristic of the envelope proteins (Smith et al., 1974; Kääriäinen et al., 1975). The 42S RNA must necessarily code for the replicative protein(s) and other essential non-structural proteins.

f) Viral effect on host cell metabolism

Host cell RNA synthesis decreases when viral RNA synthesis begins, there is no specific shut off during the latent period of viral infection (Taylor, 1965). Interferon is produced by chick embryo cells infected with SFV, it is detectable late in infection and has no marked effect on virus multiplication (Taylor, 1965). The induction of interferon is temperature-sensitive; normal viral RNA and interferon induction occurs at 36°C but not at 42°C (Skehel and Burke, 1968). The use of RNA(+) and RNA(-) temperature-sensitive mutants suggests that interferon induction is dependent on virus RNA synthesis early in infection, but that double-stranded RNA is not essential (Lomniczi and Burke, 1970; Atkins et al., 1974; Atkins and Lancashire, 1975).

5hr after infection of chick embryo cells with SFV, cytoplasmic dehydrogenase enzymes show increased activity. Thus the glucose catabolic pathways are stimulated following virus infection (Cassells and Burke, 1973). The initial increase in the activity of one enzyme, cellular lactate dehydrogenase (LDH) is followed by a decrease correlated with an increase of activity in the growth medium. Apparently the inhibitory action of virus infection causes a release of LDH by chick cells (Cassells, 1973).

The reduced phospholipid synthesis observed after infection of chick cells by Sindbis virus was suggested to be a secondary effect resulting from the shut off of host RNA and protein synthesis (Waite and Pfefferkorn, 1970b). However,

SFV, but not bovine enterovirus, causes an inhibition of choline incorporation into 3-sn-phosphatidylcholine of the cytoplasmic membrane and, also a decrease in the specific activity of CDP-choline: 1,2-diglyceride choline phosphotransferase (Vance and Burke, 1974).

80 to 90% of host protein synthesis is inhibited 2 to 3hr post-infection; Sindbis specified proteins form 60 to 70% of the total protein synthesis (Strauss, Burge and Darnell, 1969).

g) The RNA-dependent RNA polymerase

With the discovery of RNA containing viruses it was necessary to determine whether DNA was involved in the mechanism of RNA replication. SFV will replicate in enucleate cells and in the presence of actinomycin D indicating that there is no involvement with nuclear DNA during replication.

RNA-dependent RNA polymerases (RNA replicases) were isolated at about the same time from cells infected with mengovirus (Baltimore and Franklin, 1962, 1963) and from E.coli infected with MS2 (Haruna et al., 1963). Since then the replicases involved in RNA bacteriophage replication have been completely purified and extensively characterized (Stavis and August, 1970). Unfortunately, this has not yet been achieved for animal viruses.

An enzyme, isolated from alphavirus infected cells, which catalyses the incorporation of guanosine, adenine, cytidine and uridine 5'-triphosphates into an acid-insoluble, alkali-labile product was first described by Lust (1966) and Martin and Sonnabend (1967). This enzyme, an RNA replicase, is insensitive to Actinomycin D and deoxyribonuclease and is dependent on magnesium ion concentration. It has proved

difficult to isolate an enzyme capable of making all the viral RNA species. Commonly, double-stranded RNA, and a small amount of RI are synthesized (Levin and Friedman, 1971). Sreevalsan and Yin (1969) reported that an enzyme extracted from Sindbis virus infected cells synthesized, with low specific activity, single-stranded RNA. The types of RNA synthesized were not well characterized. There has been only one convincing demonstration of enzyme activity which synthesizes, in vitro, RFs, RI and 42, 26 and 22S single-stranded RNA (Michel and Gomatos, 1973). The RFs and RI are first synthesized, and the radioactive label in them can be chased into single-stranded RNA. Over 50% of the pulse-label in RFs could be chased into single-stranded RNA suggesting that one strand of the template (the negative strand) is conserved. However, by polyacrylamide gel electrophoresis, the RF synthesized in vitro appeared to be much smaller than a duplex of 42S positive and negative strands, and the authors therefore suggested that the 42S RNA must be derived from more than one RNA chain. Two observations make this unlikely, a) the many reports of the existence of RFI, and strand composition analysis which shows that it consists of a duplex of two 42S RNA molecules, and b) polyacrylamide gel electrophoresis does not give a true estimate of molecular weight of this double-stranded RNA. The enzyme preparation used by Michel and Gomatos was extremely crude; it seems that during purification of the RNA replicase either a specific double-stranded RNA synthesizing RNA replicase is isolated, or the ability of the enzyme to synthesize single-stranded RNA is lost because of the disruption of the RNA-template-membrane complex, possibly as a result of ribonuclease action.

RNA replicase activity is intimately associated with

membranes; CPV-1 isolated by isopycnic centrifugation are heavily enriched with viral RNA and RNA replicase (Friedman et al., 1972).

Aspects of the multiplication cycle have been here described separately and not necessarily in temporal sequence for reasons of clarity of presentation. Figure 2 shows a simplified version of SFV "life-cycle".

#### 4. The rationale of this work

SFV, with a particle-to-infectious virus ratio close to one (Cheng, 1961), a wide host range, rapid growth to high titres in tissue culture, and little or no pathogenicity for man (Andrewes and Pereira, 1972) is thus a good experimental agent. Together with Sindbis it is the representative alphavirus of the Togaviridae which is the main family, besides the Picornaviridae of the Class IV group of viruses in the Baltimore scheme of classification. The picornaviruses have been extensively studied, it is only recently that knowledge of alphavirus replication has become as advanced. As virion RNA is infectious, RNA replicase (which is absent in uninfected cells) must be one of the first proteins translated and is thus a target for anti-viral agents before extensive viral replication and destruction of the host cell have occurred. Obviously, before RNA replicases can be considered as targets for anti-viral agents they must be purified and characterized. The purpose of this study, therefore, was to attempt to purify the RNA replicase utilizing any method which did not destroy the activity as measured in vitro.

When this work was started there were several obvious questions: (1) Is there more than one replicase? (2) Is there any subunit structure to the replicase? (3) What is

the host's involvement in RNA transcription? (4) What is the replicase competent to synthesize in vitro? The experiments described are attempts to answer these questions, and as with any purification procedure, many dead-ends were reached, and many red-herrings were pursued.

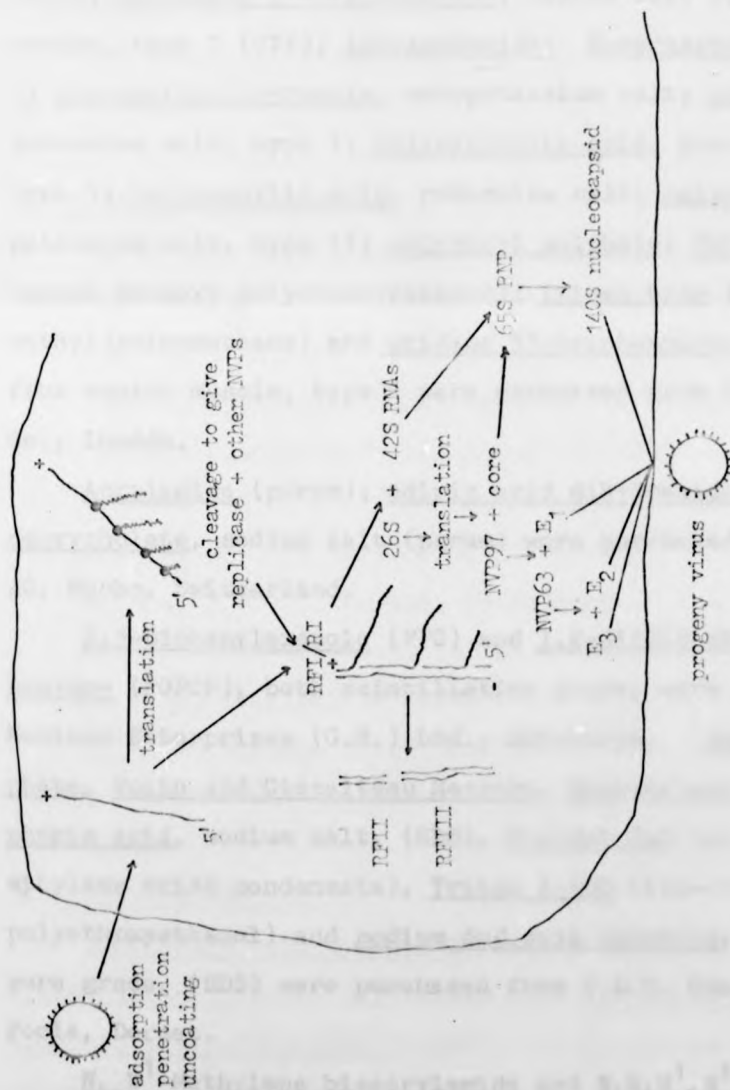


Figure 2. A possible scheme for the multiplication of SVV based on models previously suggested (Simons and Strauss, 1972b; Burke and Russell, 1975).

*Escherichia coli* (DH5α) were purchased from Gibco, Grand Island, N.Y., U.S.A.

*Deoxyribonuclease* 500 and *RNAse* 500 were purchased



## MATERIALS

### 1. Chemicals

Adenosine 5'-triphosphate, disodium salt from equine muscle (ATP); Brij 58 (polyoxyethylene 20 cetyl ether); cytidine 5'-triphosphate, sodium salt, type III synthetic (CTP); guanosine 5'-triphosphate; sodium salt from equine muscle, type 1 (GTP); iodoacetamide; 2-mercaptoethanol, type 1; phospho(enol)pyruvate, monopotassium salt; polyadenylic acid, potassium salt, type 1; polycytidylic acid, potassium salt, type 1; polyguanylic acid, potassium salt; polyuridylic acid, potassium salt, type 11; polyvinyl sulphate; Triton N-101 (nonyl phenoxy polyethoxyethanol); Trizma base (tris(hydroxymethyl)aminomethane) and uridine 5'-triphosphate, sodium salt from equine muscle, type 1 were purchased from Sigma Chemical Co., London.

Acrylamide (purum); adipic acid dihydrazide (pract.) and deoxycholate, sodium salt (purum) were purchased from Fluka AG, Buchs, Switzerland.

2,5-Diphenyloxazole (PPO) and 1,4-di(2(5-phenyl-oxazolyl))-benzene (POPOP), both scintillation grade, were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh. Ammonium persulphate, Folin and Ciocalteu Reagent, Naphthalene 1,5-disulphonic acid, sodium salt, (NDS), Nonidet P40 (an octylphenol ethylene oxide condensate), Triton X-100 (iso-octylphenoxy-polyethoxyethanol) and sodium dodecyl sulphate, especially pure grade, (SDS) were purchased from B.D.H. Chemicals Ltd., Poole, Dorset.

N, N<sup>1</sup>-Methylene bisacrylamide and N, N, N<sup>1</sup>, N<sup>1</sup>-tetramethylene-diamine (TEMED) were purchased from Eastman Kodak Co., Rochester, New York, U.S.A.

Dextran sulphate 500 and CNBr-Sepharose 4B were purchased

from Pharmacia, London.

Oligo (dT)-cellulose, type T3 was purchased from Collaborative Research Inc., Waltham, Mass., U.S.A.

Affi-Gel 10, N-hydroxysuccinimide derivative of Bio-Gel A was purchased from Bio-Rad Laboratories, Bromley, Kent.

Cleland's Reagent (dithiothreitol) grade A was purchased from Calbiochem, Ltd., London.

All other chemicals were the best grade available commercially.

## 2. Radio-labelled Compounds

These were purchased from the Radiochemical Centre, Amersham, Bucks.

[2-  $^3\text{H}$ ] Adenosine 5'-triphosphate, 20 Ci/mmol.

[5-  $^3\text{H}$ ] Cytidine 5'-triphosphate, 20 Ci/mmol.

[8-  $^3\text{H}$ ] Guanosine 5'-triphosphate, 12 Ci/mmol.

Guanosine 5'-[ $\alpha$  -  $^{32}\text{P}$ ] triphosphate, 1.04 - 3.66 Ci/mmol.

L - [2, 5 -  $^3\text{H}$ ] Histidine, 55 Ci/mmol.

L - [4, 5 -  $^3\text{H}$ ] Leucine, 53 Ci/mmol.

L - [ $^{35}\text{S}$ ] Methionine, 240 Ci/mol.

[5 -  $^3\text{H}$ ] Uridine, 25 Ci/mmol.

[5 -  $^3\text{H}$ ] Uridine 5'-triphosphate, 25 Ci/mmol.

L - [2,3 -  $^3\text{H}$ ] Valine, 17.7 Ci/mol.

## 3. Biological Methods

Pancreatic ribonuclease A, type X11-A (RNase A), ribonuclease T<sub>1</sub>, grade III (RNase T<sub>1</sub>), cycloheximide and albumin, bovine fraction V (BSA) were purchased from Sigma Chemical Co., London.

Actinomycin D was a generous gift from Merck, Sharpe and Dohme Research Laboratories, N.J., U.S.A.

Deoxyribonuclease, ribonuclease-free, electrophoretically

purified, (DNase) was purchased from Worthington Biochemical Corp., N.J. U.S.A.

Pyruvate kinase, a suspension in glycerol, was purchased from Boehringer Mannheim GMBH, Germany.

Medium 199 and Minimal Essential Medium, Glasgow modification (GMEM) were purchased from Wellcome Reagents, Ltd., Beckenham, Kent.

Calf serum was purchased from Bio-cult Laboratories Ltd., Paisley, Scotland.

Earle's salt solution, Brain Heart Infusion (BHI) and Nutrient Broth were purchased from Pharmex, Ltd., Bexley, Kent.

Crystamycin (containing 600 mg. (1 million units) of penicillin to 1g. of streptomycin) was purchased from Glaxo Laboratories, Ltd., Greenford, Essex.

Tryptose phosphate broth, peptone and yeast extract were purchased from Difco Laboratories, East Molesley, Surrey.

Agarose was purchased from L'industrie Biologique Française S.A., Gennevillies, Seine, France.

#### 4. Tissue Culture Media

Eagle's GMEM was prepared from a ten fold concentrate and was supplemented with 10% (v/v) calf serum, 2.95 g/l tryptose phosphate broth, 100 units/ml of crystamycin and was adjusted to pH 7.4 with 5% (w/v) sodium bicarbonate solution.

Maintenance Medium was prepared from a ten fold concentrate of Medium 199 and was supplemented with 2% (v/v) calf serum, 100 units/ml of crystamycin and was adjusted to pH 7.4 with 5% (w/v) sodium bicarbonate solution.

Growth Medium was identical in content to Maintenance Medium except that the concentration of calf serum was 5% (v/v) not 2% (v/v).

Earle's salt solution containing dialysed calf serum and Actinomycin D (EDA). The salt mixture was dissolved in distilled water, autoclaved at 15 p.s.i. for 20 min, adjusted to pH 7.4 with 5% (w/v) sodium bicarbonate. Phenol red (0.001%) was used as an indicator of pH. Calf serum dialysed overnight at 4°C against Earle's salt solution (pH 7.4) was added to 2% (v/v). Actinomycin D was added to 1µg/ml.

Brain Heart Infusion B.H.I. 37g/l., autoclaved at 15 p.s.i. for 20 min. before use.

Nutrient Broth 13g/l., autoclaved at 15 p.s.i. for 20 min. before use.

YEPG was 1% (w/v) yeast extract, 1% (w/v) peptone and 1% (w/v) glucose. YEPG was autoclaved at 15 p.s.i. for 20 min. before use.

The media made up as described above were checked for sterility by separately innoculating in B.H.I. (5 days at 37°C), Nutrient Broth (5 days at 37°C) and YEPG (5 days at 30°C).

## 5. Buffers and Solutions

As a diversity of buffers and solutions were employed only those routinely used will be given here. Others will be described in the relevant section in the text.

Reticulocyte standard buffer (RSB) was 10mM-tris containing 10mM NaCl and 1.5mM MgCl<sub>2</sub> (pH 7.4). RSB was made up 5 fold concentrated, autoclaved at 15 p.s.i. for 20 min. and diluted with sterile distilled water before use.

RSBM was RSB containing 10mM 2.mercaptoethanol.

Assay buffer was 100mM-tris containing 10mM MgCl<sub>2</sub> and 1mM KCl (pH 8.0). This was made up 5 fold concentrated, autoclaved at 15 p.s.i. for 20 min. and diluted with sterile

distilled water before use.

TNE was 50mM-tris containing 100mM NaCl and 1mM ethylene diamine tetra-acetic acid (disodium salt) (pH 7.4). TNE was made up 5 fold concentrated, autoclaved at 15 p.s.i. for 20 min. and diluted with sterile distilled water before use.

TNES was TNE containing 0.1% (w/v) SDS.

SDS was kept as a 10% (w/v) solution sterilized by autoclaving at 15 p.s.i. for 20 min.

Phosphate-buffered saline (PBS) was 139mM NaCl containing 28mM KCl, 7.5mM  $\text{Na}_2\text{HPO}_4$  and 0.147mM  $\text{KH}_2\text{PO}_4$  (pH 7.4) sterilized by autoclaving at 15 p.s.i. for 20 min.

Standard saline citrate (SSC) was 150mM NaCl containing 15mM sodium (tri) citrate (pH 7.0 with HCl) sterilized by autoclaving at 15 p.s.i. for 20 min.

"Trypsin" for cell passage was 8g NaCl, 0.38g KCl, 0.1g  $\text{Na}_2\text{HPO}_4$ , 1g D-glucose, 3g tris, 15ml 1% (w/v) phenol red,  $10^5$  units of penicillin, 0.1g streptomycin and 2.5g of trypsin in 1 litre (pH 7.7). The salts, glucose, tris and phenol red were dissolved in distilled water, the pH adjusted with NaOH/HCl and the remaining components added to give 1 litre. Sterilization was by membrane filtration through 3, 0.22 micron Millipore filters (Millipore (U.K.) Ltd., London) under a positive pressure atmosphere of nitrogen.

"Versene" for cell passage was 8g NaCl, 0.2g KCl, 1.15g  $\text{Na}_2\text{HPO}_4$ , 0.2g  $\text{KH}_2\text{PO}_4$ , 0.2g EDTA, 1% (w/v) phenol red in 1 litre of distilled water sterilized by autoclaving at 15 p.s.i. for 20 min.

Sucrose solutions were made up in the appropriate 5 fold concentrate buffer and were sterilized by autoclaving at 10 p.s.i. for 10 min. For velocity gradient centrifugation,

solutions were w/v, whilst for isopycnic centrifugation solutions were w/w.

#### 6. Scintillation mixtures

Toluene scintillation mixture was 8g of PPO in 2 litres of toluene.

Toluene-triton scintillation mixture was 1 volume of Triton X-100 and 2 volumes of toluene scintillation mixture containing 0.266 g/l final concentration of POPOP.

#### 7. Purification of reagents

Ethanol, ether and phenol were redistilled before use. Acrylamide and N,N'-methylene bisacrylamide were recrystallised before use from chloroform and acetone respectively (Loening, 1967). Commercial [ $^3\text{H}$ ] GTP was degassed under vacuum for 20 sec. to remove excess ethanol which was reported to inhibit an RNA polymerase from Micrococcus lysodeikticus, (Straat, Ts'o and Bollum, 1968).

## METHODS

### 1. Subculturing of continuous cell lines

Baby hamster kidney (BHK) cells, were routinely used in this work. On occasion rabbit kidney (RK) cells were used and were passaged in the same way. Cells were grown in 14cm plastic Petri dishes or 2.5 l smooth-walled roller bottles.

To prepare cells for seeding the fluid from a roller bottle was poured off and the cell sheet washed twice with 12ml versene/trypsin (4:1 v/v)). The bottle was then incubated at 37°C until the cells had detached from the glass (3-5 min.) at which time 10ml of GMEM was added. The cells were dispersed by pipetting and counted using a Neubauer haemocytometer. Cells were seeded at  $5 \times 10^6$  cells in 200ml of GMEM for roller bottles, and  $10^6$  cells in 25ml of GMEM for plastic Petri dishes. The cells were then incubated at 37°C until the monolayer reached confluence. Before incubation the bottles were gassed for 20 seconds with a 5% CO<sub>2</sub>/95% air mixture. Cells grown on plastic Petri dishes were maintained in a humid atmosphere of 5% CO<sub>2</sub>/95% air. At confluency the 14cm plastic Petri dishes contained  $\sim 8 \times 10^7$  cells and the roller bottles  $\sim 3 \times 10^8$  cells.

### 2. Preparation of chick embryo cells (CEC)

The method used was that of Morser, Kennedy and Burke, (1973). Embryos from 11 day old hen's eggs were removed, decapitated and chopped by hand using scissors. The minced torsos were repeatedly suspended in 0.1% trypsin in PBS at 37°C for 20 min. The supernatant fluids were combined, filtered through a 40 mesh stainless steel gauze, pelleted by centrifugation, resuspended in medium 199 containing 5% (v/v) calf serum and 100 units/ml each of penicillin and streptomycin,

and finally passed through a grade 0 then a grade 1 sintered glass filter. Suspensions were diluted in Medium 199 to the desired concentration, seeded on 14cm plastic Petri dishes and incubated at 37°C in 5% CO<sub>2</sub>/95% CO<sub>2</sub> before use. Seeding was in 20ml of medium containing  $7.5 \times 10^6$  cells/ml. At confluency there were  $\sim 10^8$  cells in a monolayer.

### 3. Growth of SFV.

SFV stock was plaque purified virus originally obtained from Dr. J. Porterfield, National Institute of Medical Research, Mill Hill, London.

#### a) Growth in brains of suckling mice

The virus was grown as described by Walters, Burke and Skehel (1967).

#### b) Growth in suspension culture

The virus was grown in suspension cultures of chick embryo cells using the modification of the method of Zwartov and Algar (1968) as described by Kennedy and Burke (1972). A suspension of primary chick embryo cells was infected with plaque purified mouse brain grown SFV at a m.o.i. of 1 for 1hr. at 4°C. The infected cells were diluted to  $2$  to  $3 \times 10^7$  cells/ml. with Earle's medium containing 0.35% (w/v) glucose, 0.4% (w/v) sodium bicarbonate, 0.001% (w/v) phenol red and 180 units/ml. of colomycin. The pH was adjusted to 7.5 by bubbling with 90% O<sub>2</sub>/10% CO<sub>2</sub>. The suspension was dispersed, the atmosphere displaced with 95% O<sub>2</sub>/5% CO<sub>2</sub> and the flasks incubated at 35°C on an orbital shaker moving at 80 rev/min. After 18hr. the suspension was clarified by centrifugation at 15,000g for 30 min. at 4°C and the supernatant containing  $2 \times 10^9$  to  $10^{10}$  p.f.u./ml. of virus was supplemented with 5% (v/v) calf serum and stored at -70°C. This virus was used as innoculum throughout this work.



#### c) Growth of SFV in BHK cells

The fluids were removed from confluent monolayers of BHK cells grown in roller bottles, washed once with maintenance medium and infected with virus at a m.o.i. of 1 for 1hr at 37°C. The fluids were then removed, replaced with 50ml. of maintenance medium and incubated at 37°C. After 22½hr. the fluids were removed, clarified by centrifugation at 15,000g for 30 min. and the supernatant either used immediately or stored at -70°C.

#### 4. Purification of SFV

Virus grown either in suspension culture of chick embryo cells or in BHK cells was concentrated using a Bio-Rad ultrafiltration unit (cellulose acetate-C hollow fibres, Bio-Rad Laboratories, Richmond, California). The concentrated fluids, supplemented with 0.5% (v/v) calf serum, were layered onto a 28ml 20 to 55% (w/v) sucrose gradient in TNE supplemented with 0.5% (v/v) calf serum. Centrifugation was for 17 to 19hr in an MSE 3 X 65ml swing-out rotor at 75,000g (4°C). The virus formed an opalescent band approximately two thirds of the way down the gradient. This was recovered by piercing the tube at the bottom using an MSE tube piercer. The virus was then diluted in TNE and pelleted by centrifugation at 120,000g for 2½hrs in an MSE 8 X 25ml angle rotor (4°C). The virus pellet was resuspended in a few ml of TNE using a loose-fitting glass Dounce homogeniser.

#### 5. Preparation of radiolabelled SFV

In order to grow radioactively labelled virus, the isotope, either [<sup>3</sup>H] uridine or [<sup>35</sup>S] methionine was added to the medium to 10 µCi/ml after 1hr virus adsorption. When virus RNA was

being labelled Actinomycin D was added after 1hr virus adsorption to 0.1  $\mu\text{g/ml}$ .

#### 6. Preparation of SFV nucleocapsids

SFV nucleocapsids (150S) were used as a marker on certain gradients. Following the method of Kääriäinen and Söderlund (1971) purified SFV was suspended in 50mM-tris, 100mM NaCl (pH 7.4) and an equal volume of 2% (v/v) Nonidet P40 in water added. After incubation at room temperature for 30 min. the nucleocapsid preparation was analysed on a gradient. Figure 3 shows the sedimentation of a typical preparation.

#### 7. Infection of monolayer cultures

For experiments in which RNA replicase activity was measured monolayers of cells were washed with maintenance medium, infected with SFV at a multiplicity of 25-100 p.f.u./cell and incubated at 37°C. After 1hr the unadsorbed virus was removed and the cells incubated with 50ml maintenance medium. After a further 2hr the fluids were replaced with 50ml maintenance medium containing 1  $\mu\text{g/ml}$  of Actinomycin D. The cells were harvested 2hr later by means of a specially constructed cell scraper. This protocol was varied for time course experiments and for experiments in which protein or RNA was labelled early in infection.

Mock infected cultures were treated in the same manner except that maintenance medium was added instead of virus.

#### 8. Preliminary fractionation of infected cell cultures

CEC, RK cells and BHK cells were fractionated in the following manner: The medium was removed and the cell mono-

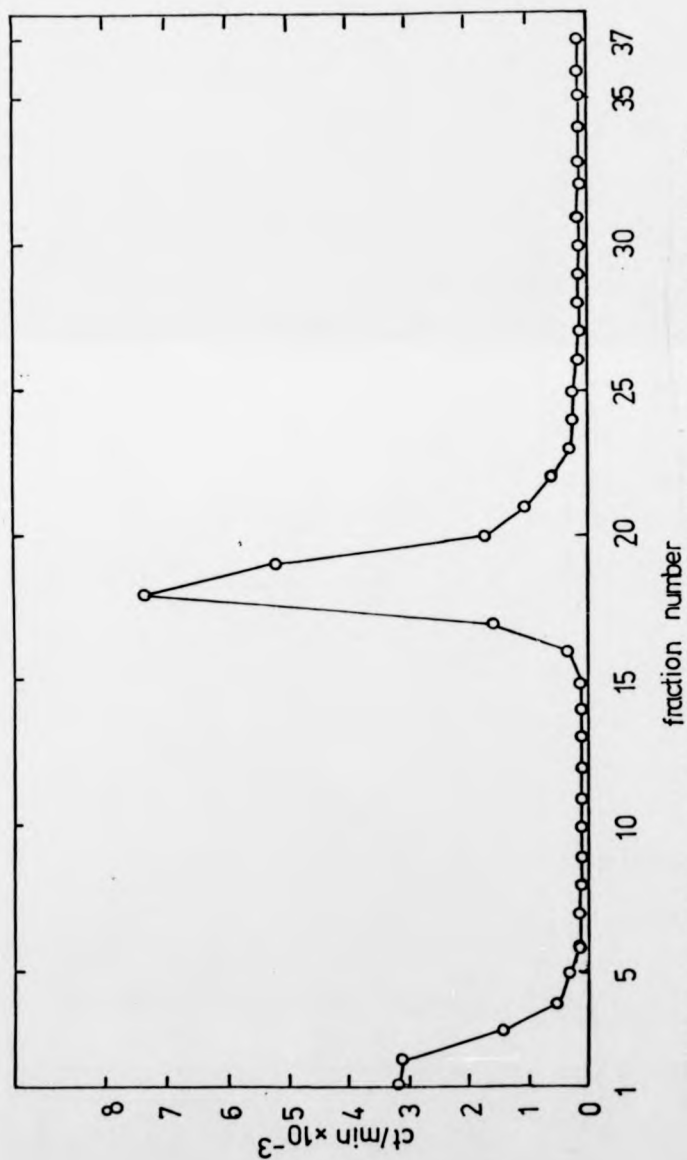


Figure 3. Sucrose gradient sedimentation of SFV nucleocapsids. 100,000 ct/min of [ $^3\text{H}$ ]-uridine labelled SFV, grown in suspension culture and purified by ultrafiltration and sucrose gradient centrifugation, was treated with Nonidet P40 as described in Methods. The treated sample was layered on a 5-65% (w/v) linear sucrose gradient in RSEM containing 1% (v/v) Triton N-101 and centrifuged at 40,000 rpm for  $3\frac{1}{2}$  hr at  $4^\circ\text{C}$  in an M.S.E. 6x14 ml swing-out rotor. Fractions were collected by pumping off from the top of the gradient, and radioactivity determined. In this, and all subsequent figures of sucrose gradients, the top of the gradient is on the left.

layer washed 4 times with ice-cold PBS. The cells were recovered by scraping off the glass, pipetted into sterile universal bottles and recovered by centrifugation at 600g for 5 min using an MSE bench centrifuge in a cold room. All subsequent operations were performed at 4°C using sterile apparatus and solutions. The supernatant PBS was decanted and at this stage the cells could be stored at -70°C for 3 months without loss of RNA replicase activity. The cells were resuspended in RSBM by gentle swirling for 15 min. ( $3 \times 10^8$  cells/ml.) and then disrupted with 28 strokes of a stainless steel Dounce homogeniser having a clearance of 0.002in. for 4in. of the barrel length. The cell lysate was centrifuged at 700g for 10min to remove unbroken cells, nuclei and cell debris. The supernatant was recovered and centrifuged at 15,000g for 20 min. The supernatant (S15) was discarded and the pellet resuspended in RSBM to give a protein concentration of approximately 1mg/ml. This is referred to as P15.

9. Isopycnic centrifugation of cytoplasmic extracts in discontinuous sucrose-density gradients

Isopycnic centrifugation separates rough and smooth cytoplasmic membranes. This method is based on that described by Caliguiri and Tamm (1970a) and Friedman *et al* (1972). The gradient, prepared in a 25ml polycarbonate centrifuge tube consisted of 3ml of 65% sucrose, 4ml of 45% sucrose, 4ml of 40% sucrose, 5ml of 30% sucrose containing P15 from BHK cells, 4ml of 25% sucrose and 3ml of RSBM. All sucrose solutions were w/w in RSBM. Centrifugation was in a MSE 3 X 25ml swing out rotor at 96,000g for 17 to 19hr at 4°C. As will be described in the results section several bands were visible.

Fractions were collected by piercing from the bottom or by using a "Densi-flow" (Buchler Instruments, Baird and Tatlock, London) and pumping off from the top. The density of fractions was determined using an Abbey refractometer (Bellingham and Stanley, London). Membrane bands were recovered by diluting with RSBM and centrifuging at 50,000g for 1hr at 4°C.

#### 10. Linear velocity sucrose-gradient centrifugation

Samples containing protein were analysed on linear gradients of sucrose prepared in RSBM buffer. RNA was analysed on gradients prepared in TNES. Gradients were formed at room temperature and allowed to equilibrate at the running temperature. Gradients were run at 4°C unless SDS was present in which case the temperature was 15°C. Gradients were unloaded by upward displacement with 70% (w/v) sucrose or by using the "Densi-flow" and were either collected into an LKB fraction collector (LKB Instruments, Croydon, Surrey) or a Gilson fraction collector (Anachem Ltd., Luton, Beds.)

#### 11. Assay for RNA dependent RNA-polymerase activity

The reaction mixture was 250µl final volume (excluding 100µl of sample) and contained the following in Assay buffer:

Energy generating system: pyruvate kinase (PK) 20µg (0.3 enzyme units) phospho(enol)pyruvate (PEP) 5 µmoles.

Nucleotides: ATP, CTP and UTP 50nmoles each. GTP 1nmole, 2.5 µCi as [<sup>3</sup>H]

Reducing agent: Cleland's reagent (dithiothreitol, DTT) 1.5 µmoles.

Ribonuclease inhibitor: Dextran sulphate 500, 3.5µg.

To prevent DNA dependent RNA-transcription: Actinomycin D, 1µg.

, KCl: 4.0 µmoles.

The above reagents were prepared as follows and were stored at  $-20^{\circ}\text{C}$  in 1ml aliquots.

- (i) PK : 0.2ml of Boehringer 10mg/ml PK was diluted to 5ml with Assay buffer.
- (ii) PEP : 0.104g was dissolved in 5ml of Assay buffer.
- (iii) 0.0276g ATP, 0.0264g of CTP and 0.0288g of UTP were dissolved in 5ml of Assay buffer, the pH checked, adjusted if necessary to pH 8.0 with sterile 1N NaOH and 0.5ml diluted to 5ml with Assay buffer.
- (iv) 0.296g of GTP was dissolved in 5ml of Assay buffer, the pH checked, adjusted if necessary to pH 8.0 with sterile 1N NaOH, and 10 $\mu$ l diluted to 5ml with Assay buffer.
- (v) Actinomycin D, DTT, KCl and Dextran sulphate 500:  
0.1ml of Actinomycin D at 1mg/ml was mixed with 1.0ml of DTT at 0.023g/ml in Assay buffer, 1.0ml of sterile KCl at 0.298g/ml in Assay buffer, 0.1 of Dextran sulphate 500 at 0.0035g/ml in Assay buffer and made to 5.0ml with Assay buffer.

In order to assay for RNA replicase activity equal volumes of solutions (i), (ii), (iii), (iv) and (v) were mixed at  $4^{\circ}\text{C}$ . Solution (iv) (GTP) was first transferred to a sterile round bottom flask, [ $^3\text{H}$ ] -GTP added (100 $\mu$ l [ $^3\text{H}$ ] -GTP per 1ml of stock GTP) and degassed under a vacuum to remove ethanol. 250 $\mu$ l aliquots of the reaction mixture were dispersed into conical test tubes on ice and 100 $\mu$ l of sample added. (The protein concentration of the sample was never greater than 1mg/ml). Where necessary the reaction volume was altered. This will be indicated at the appropriate place in the text. The reaction mixture was incubated at  $35^{\circ}\text{C}$  for 30min (unless otherwise indicated). The reaction was stopped by adding 3.5ml of ice-cold 6.5% (w/v) TCA containing 100mM tetrasodium pyrophosphate

(TCA/PP) and 0.5ml of freshly prepared 0.5mg/ml BSA in  $H_2O$ . The mixture was then left to flocculate on ice for at least 30 min. The samples were then transferred to a Millipore multifiltration apparatus (Millipore (UK) Ltd., London) and filtered under suction through Whatman GF/C filter discs (Griffin and George, Ltd., London). The filter discs were washed 5 times with TCA/PP (5ml per wash), 3 times with an ethanol/ether mixture (3:1 (v/v)) (5ml per wash) and finally with ether (2 washes each of 5mls). The discs were dried under an infra-red lamp, transferred to scintillation vials, 5ml of toluene scintillator added, cooled and radioactivity determined.

TCA precipitation of samples from sucrose gradients or affinity columns was carried out in the same manner. If the incorporation of radiolabelled amino acids into TCA insoluble material was being investigated the TCA contained 1mM cold amino acid (e.g. methionine) instead of pyrophosphate.

## 12. In vivo RNA radiolabelling

### a) Virus-specified RNA

This method is based upon that of Levin and Friedman (1971) and Martin and Burke (1974). Roller bottles of BHK cells were infected as described. Immediately prior to labelling the maintenance medium was removed, the cell sheet washed with EDA then 10ml of EDA added containing 500 $\mu$ Ci [ $^3H$ ] -uridine. Incorporation was stopped by rapidly cooling the bottles to 4°C and the cells were harvested as described. Actinomycin D was present at 1 $\mu$ g/ml for 5½hrs prior to the addition of the label. Thus it was necessary to pre-incubate the cells with Actinomycin D in the medium if the label was added prior to 4½hr. after the 1hr. virus adsorption time.

#### b) Cellular RNA

Sub-confluent BHK cells grown in a roller bottle were incubated with 100 $\mu$ Ci of [ $^3$ H] -uridine for 18-24hr. at which time the cells had reached confluency. The cells were washed with ice-cold PBS, then ice-cold TNE, scraped into universals, recovered by centrifugation and the pellet resuspended in sterile TNE.

#### 13. In vivo protein labelling

The procedure was the same as that described for labelling virus-specified RNA except that radiolabelled amino acids were added at 750 $\mu$ Ci per roller bottle. Mock infected cultures were treated in the same way as infected cultures except that maintenance medium was added instead of virus.

#### 14. Extraction of RNA

##### a) From purified virus

The virus suspension was made 2% (v/v) with respect to 2-mercaptoethanol, an equal volume of phenol saturated TNE added, then SDS added to 2% (w/v). After shaking this mixture gently the phases were separated by centrifugation at 1000g for 10min. The upper aqueous phase was kept and the lower phenol phase re-extracted with TNE containing 2% (w/v) SDS. The aqueous washes were pooled and residual phenol removed by extracting with an equal volume of a chloroform/octanol mixture (24:1 (v/v)). After centrifugation the upper aqueous phase was removed, an equal volume of ether added, the mixture gently shaken, centrifuged and the upper ether layer removed under vacuum. Residual traces of ether were removed by evaporation under a stream of nitrogen. RNA was precipitated by the addition of 2½ volumes of redistilled ethanol, overnight, at



-20°C.

b) From other preparations

The preparation was suspended in TNE, NDS added to 0.5% (w/v), SDS to 2% (w/v), then an equal volume of phenol saturated TNE added. The mixture was shaken vigorously and the phases separated by centrifugation as described above. The phenol phase was re-extracted twice with TNE. The pooled aqueous phases were extracted once with chloroform/octanol, twice with ether and precipitated as described above.

15. Nuclease digestion

a) RNase

(i) Purified RNA: The purified RNA was recovered from ethanol by centrifugation and resuspended in 80µl of TNE. A  $\frac{1}{4}$  volume of 2M NaCl, 50mM MgCl<sub>2</sub> was added followed by 4µl of a mixture of pancreatic and T<sub>1</sub> RNases (final concentrations 4µg/ml and 8 units/ml respectively). This solution was incubated for 30min at 37°C, cooled to 4°C then 10µl 10% (w/v) NDS added to stop the digestion.

(ii) Replicase assay mixture: An equal volume of 1M NaCl; 20mM tris, 20mM MgCl<sub>2</sub> (pH 7.2) containing 20µg/ml of RNase A and 100 units/ml of RNase T<sub>1</sub> was added to the assay mixture and incubated at 37°C for 30min. The reaction was stopped by added TCA and collecting TCA insoluble material on glass fibre filter discs as described or by phenol extracting the RNA.

b) DNase

Nuclear fractions from cell monolayers contained much DNA and so were very viscous. DNA was digested to reduce viscosity by suspending the material in nuclease buffer (10mM tris, 10mM MgCl<sub>2</sub>, 1mM EDTA, 100 mM NaCl (pH 7.2) adding DNase

to 50µg/ml and incubating at 37°C for 15min with agitation. SDS was added to 1% (w/v) and the RNA extracted once with phenol saturated TNE, once with chloroform/octanol and twice with ether. RNA was precipitated with 2½ volumes of ethanol overnight at -20°C.

#### 16. Preparation of protein samples for analysis on polyacrylamide gels

##### a) In the absence of sucrose

Ammonium bicarbonate was added to the protein samples to 100mM then 9 volumes of cold (-20°C) acetone added and the mixture left at -20°C for at least 30min. The protein and salt were recovered by centrifugation at 1,000g for 15min. The pellet was washed once with ethanol/ether (3:1 (v/v)), once with ether and dried at 37°C. The pellet was dissolved in 50mM tris (pH 8.5), SDS added to 2% (w/v), 2-mercaptoethanol to 1% (v/v), boiled for 2½min. then made 0.3M with respect to iodoacetamide and incubated at 37°C for 30min. The samples were dialysed overnight against 40mM boric acid, 40mM tris, 0.1% (w/v) SDS. If the samples were frozen at this stage they were boiled for 90 sec immediately before loading onto a gel.

##### b) In the presence of sucrose

Sucrose interferes with the precipitation of proteins by acetone so any samples containing sucrose were prepared using ethanol in the place of acetone. Ammonium bicarbonate was not added. Otherwise the procedure was the same.

#### 17. Electrophoresis of RNA on polyacrylamide gels

2.0% gels were prepared as described by Martin and Burke (1974). The gels contained 0.5% agarose. The gel components were mixed in the following order:

<u>Gel components</u>	<u>Final concentration</u>
(i) 5mls 15% (w/v) recrystallised acrylamide containing 0.75% w/v) N,N <sup>1</sup> -methylenebisacrylamide	2% 0.1%
(ii) 7.5mls of 5 fold concentrate E buffer:	
21.75g tris	36mM
23.4g NaH <sub>2</sub> PO <sub>4</sub>	40mM
1.85 EDTA	0.2mM
in 1 litre.	
(iii) 6.25ml distilled water.	
(iv) To the above, equilibrated to 50°C, was added 18.75ml 1% (w/v) molten (50°C) agarose. After degassing under vacuum for ½-1min the flask was returned to 50°C.	0.5%
(v) 12.5µl TEMED	0.033%
(vi) 0.125ml of 10% (w/v) ammonium persulphate	0.033%

After the addition of the ammonium persulphate, the gels were cast in 20cm X 6mm internal diameter perspex tubes sealed at the bottom ends with Parafilm and left to polymerise at room temperature for 1hr. When set the gels were kept at 4°C. Before use the sealing membrane was removed, the gel gently extruded using a rubber teat, the top 0.5cm removed, the tube sealed with moist dialysis membrane held in place with a rubber grommet and assembled in the gel apparatus. The sealing

membrane was pierced to minimise endo-osmosis. Gels were pre-electrophoresed at 100V. for about 30min to remove ammonium persulphate.

Electrophoresis was at room temperature at 6V/cm for 12hr. The gel tubes were immersed for most of their length in E buffer containing 0.2% (w/v) SDS. The RNA was suspended in  $\frac{1}{2}$  strength E buffer, SDS added to a final concentration of 0.2% (w/v), 5 to 10% (w/v) sucrose and about 0.01% (w/v) bromophenol blue. After electrophoresis the gels were frozen at  $-20^{\circ}\text{C}$  and fractionated into 1mm slices using a gel slicer (Mickle Laboratory Engineering Co. Ltd., Comshall, Surrey) modified so that methanol at  $-15^{\circ}\text{C}$  could be circulated through the supporting platform. Fractions were dissolved in 0.2ml hydrogen peroxide (100 volumes) at  $70^{\circ}\text{C}$ , and radioactivity determined in toluene-triton scintillator.

#### 18. Electrophoresis of proteins on polyacrylamide gels

Electrophoresis was performed using a slab gel (16 X 15 X 0.17cm) having 12 sample slots. The slab gel apparatus was constructed essentially as described by Studier (1973). The gels (Laemmli, 1970) were 8% acrylamide with a stacker gel and were cast using the following stock solutions:

1. 30% (w/v) acrylamide containing 0.8% (w/v) N,N'-methylene-bisacrylamide.
2. 10% (w/v) SDS.
3. 3M tris, 0.46% TEMED (pH 8.8)
4. 10% (w/v) freshly prepared ammonium persulphate
5. 0.5M tris, 0.4% SDS (pH 6.8) (for stacker gel)

Final ConcentrationsRunning gel

8% (w/v) acrylamide  
0.2% (w/v) N,N'-methylenebis-acrylamide  
375 mM tris  
0.057% TEMED  
0.1% SDS  
0.083% ammonium persulphate

Stacker gel

4.5% (w/v) acrylamide  
0.12% (w/v) N,N'-methylene-bisacrylamide  
0.125 M tris  
0.1% TEMED  
0.03% ammonium persulphate

Electrophoresis buffer

5mM tris  
192mM glycine  
0.1% (w/v) SDS

The sample was layered on in sucrose and bromophenol blue as described for RNA gels, electrophoresis was at 60V (constant voltage) for 16hr. After electrophoresis the slab gels were dried down under vacuum onto Whatman 3M filter paper between silicone rubber sheets at 90°C for 1hr and autoradiographed on Kodirex X-ray film.

### 19. Preparation of Immobilized ATP

a) ATP linked via 2' and 3' carbons of the ribose group to Sepharose 4B was prepared according to the method of Lamed, Levin and Oplatka (1973). The exact nature of the linkage is not known. Cyanogen bromide activated Sepharose 4B was rehydrated according to the makers' instructions and was stirred for 16hr at 4°C with a saturated solution of adipic acid dihydrazide in 100mM NaHCO<sub>3</sub> (pH 9.5) to give Sepharose adipic acid dihydrazide. ATP, oxidized by sodium periodate, was stirred for 2hr at 4°C with the washed Sepharose-adipic acid dihydrazide suspended in 200mM sodium acetate (pH 5.0). The gel was filtered, the filtrate being retained to give a measure of coupling of oxidized ATP, (determined by absorbance at 259nm measured on an Unicam SP800 spectrophotometer). The gel was stored in distilled water at 4°C. Figure 4 shows the absorbance from 220nm to 330nm for the filtrate and the oxidized ATP indicating that 40% of the oxidized ATP bound to the Sepharose.

b) N<sup>6</sup>-(6-aminohexyl)-ATP was prepared using Affi-Gel which is agarose bearing a substituted carboxyl group attached via an aliphatic arm. 2ml of 400mM ATP in 100mM NaHCO<sub>3</sub> (pH 8.5) was stirred with 0.2g Affi-Gel at 4°C. After 2hr the gel was washed with 100mM NaHCO<sub>3</sub> (pH 8.5) to remove unattached ATP. The coupling was very low (0.01%) possibly because of the unreactivity of the 6 amino group of ATP due to ionic repulsion between ATP and the matrix. Other compounds containing amino groups (e.g. ethanolamine, glucosamine and amino acids) couple with much greater affinity (Dr. J. Bennett, personal communication).

ATP-affinity columns were 30 X 6mm maintained at 0°C by circulation of ice-water through a cooling jacket. Columns

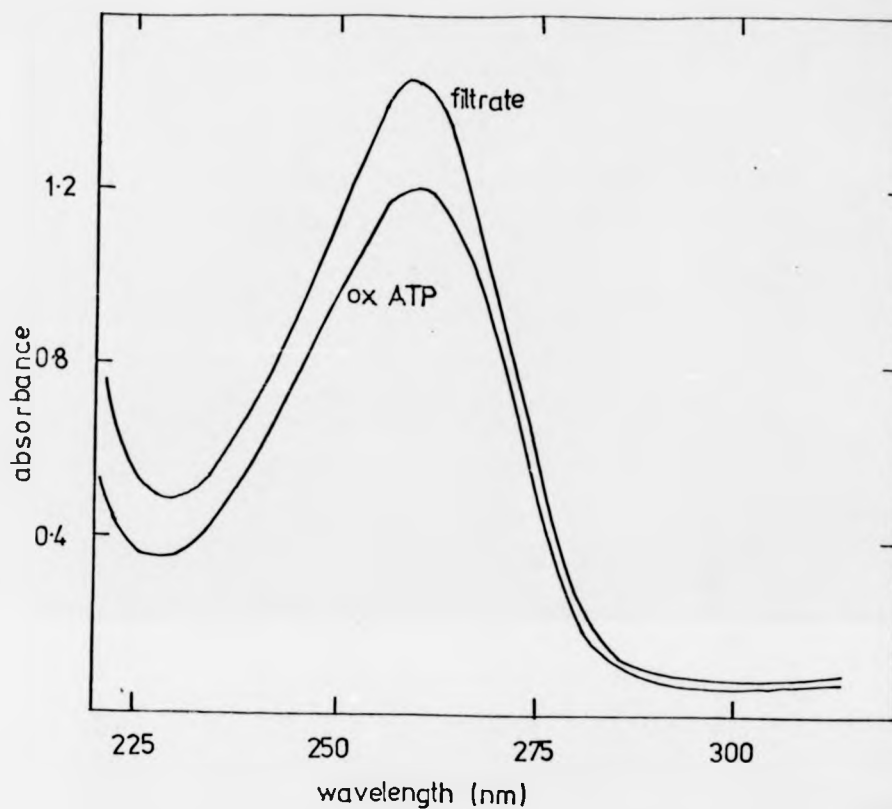


Figure 4. Determination of the percentage coupling of ATP to Sepharose. The absorbance of a sample of the filtrate collected after coupling of oxidized ATP to the Sepharose-adipic acid dihydrazide was measured on a Unicam SP800 spectrophotometer over the wavelength range shown. A sample of the oxidized ATP was similarly analysed. After correcting for dilution (the oxidized ATP sample was twice as dilute as the filtrate sample) it was found that 40% of the oxidized ATP bound to the Sepharose. This represents about 40  $\mu$ moles of ATP per 5g of Sepharose.

were washed before use at 1.5ml/min with 10ml RSBM containing 10 $\mu$ g/ml Dextran sulphate 500 and with 10ml RSBM. The sample was run in at 0.6ml/min, non-binding material was washed off with RSBM at 1.5ml/min. Bound material was recovered by eluting with RSBM containing 10mM ATP, and where indicated, with RSBM containing 500mM KCl.

#### 20. Oligo(dT)-cellulose columns

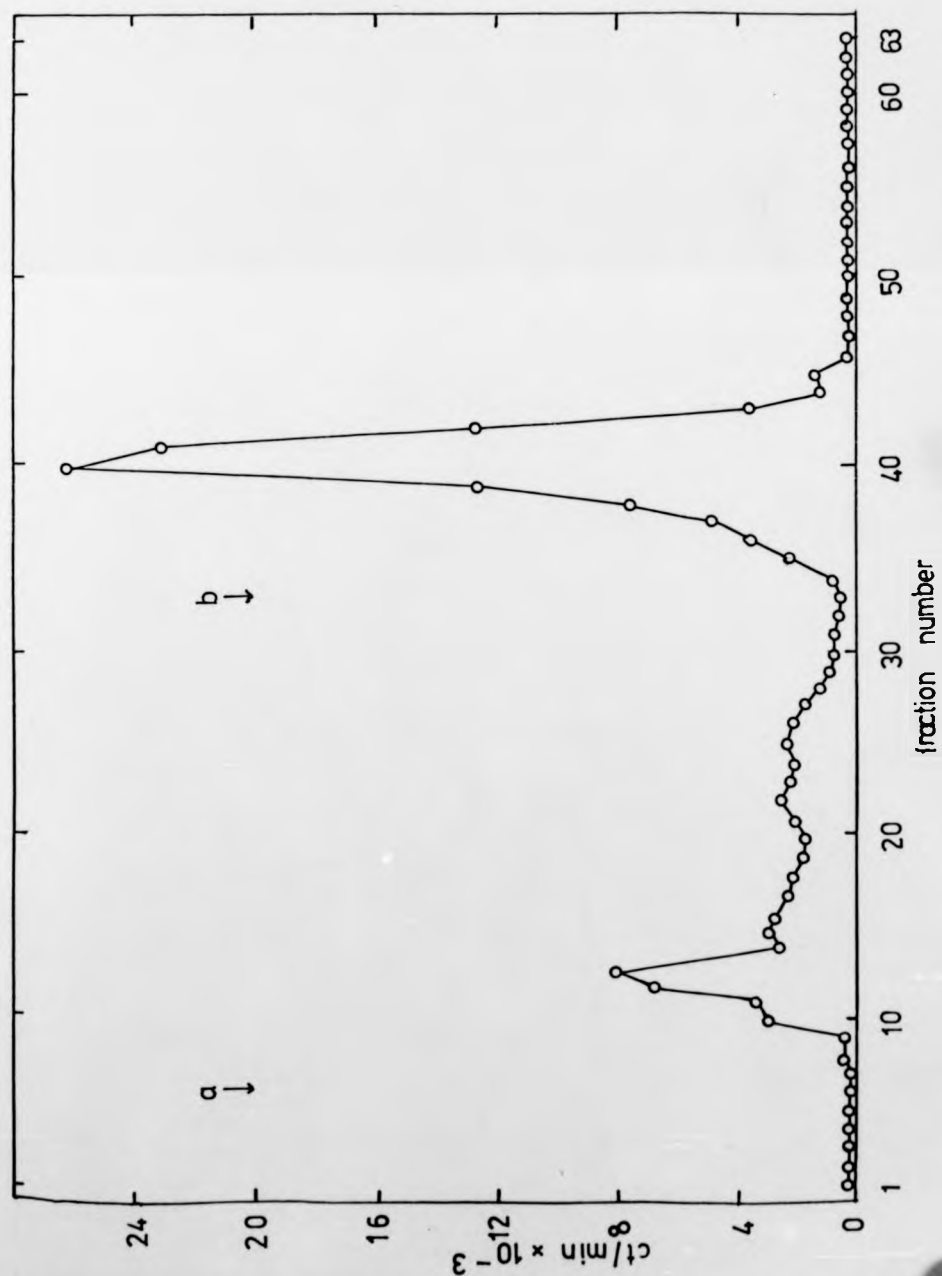
The procedure was adapted from that described by Clegg and Kennedy (1974). Oligo(dT)-cellulose was resuspended in elution buffer (10mM tris, 0.1% (v/v) Triton N-101 (pH 7.5)) at 30°C, transferred to the column (30 X 6mm) and washed with 20ml of binding buffer (50mM tris, 100mM NaCl, 1mM EDTA, 0.1% (v/v) Triton N-101 (pH 7.5)). The sample was made 100mM NaCl by adding 4M NaCl and passed at 0.6ml/min through the column. Non-bound material was removed by passing binding buffer through the column at 1.5ml/min at 30°C. Material that bound to the column was eluted at room temperature by elution buffer flowing through the column at 1.5ml/min. Under these conditions 70% of a [<sup>3</sup>H] -labelled sample of SFV 42S virion RNA bound (Figure 5).

#### 21. Preparation of poly(C)-Sephadex.

This method was taken from Yogo and Wimmer (1973) and Wagner, Bugianesi and Shen (1971). 1.5g of CN-Br-activated Sephadex 4B was washed with 200ml of 1mM-HCl and 50ml of 0.1M NaHCO<sub>3</sub> in 0.5M NaCl, pH 8.0. 25mg of Sigma poly(C) was added to the slurry and rolled at room temperature for 1hr and then overnight at 4°C. The active groups were blocked using 0.5M glycine for 5hr at 4°C. The gel was then washed



Figure 5. Chromatography of NFV virion 42S RNA on an oligo(UT)-cellulose column. [<sup>3</sup>H]uridine labelled virion RNA was prepared as described in Ref. 1. The RNA was run into the column in 100 mM NaCl at 0°C. Arrow a indicates the point at which the column was allowed to come to room temperature (20°C). Non-bound RNA was eluted by 50 mM Tris, 100 mM NaCl, 0.1 M NaOH, 0.1% (v/v) Triton X-101 (pH 7.5). Arrow b indicates when the temperature was raised to 30°C and bound RNA eluted by 100 mM Tris, 0.1% (v/v) Triton X-101 (pH 7.5). The collectivity of the fractions was determined.



with 300ml of 0.1M acetate in 0.5M NaCl (pH 4.0), then 300ml of 0.1M borate in 0.5M NaCl, pH9.0. Finally the gel was washed with PBS and stored at  $-20^{\circ}\text{C}$  in 50% glycerol.

## 22. Protein determinations

Protein concentrations were determined by the method of Lowry et al., (1951). Bovine serum albumin (fraction V) was used as standard. If the sample contained sucrose standards were determined in the presence of the same concentration of sucrose. If the sample contained Triton N-101, standards were measured in the same concentration, the detergent being removed by centrifugation prior to the measurement of optical density on a spectrophotometer.

## 23. Measurement of radioactivity in samples containing more than one isotope

A model 3358 Packard Tri-Carb Liquid Scintillation Spectrophotometer (Packard Instruments, Ltd., Middlesex) was used. This machine has channel selection facilities to allow [ $^{14}\text{C}$ ]-radioactivity to be measured in the presence of [ $^3\text{H}$ ]-radioactivity, [ $^3\text{H}$ ]-radioactivity to be measured in the presence of [ $^{14}\text{C}$ ]-radioactivity and [ $^{32}\text{P}$ ]-radioactivity to be measured in the presence of [ $^{14}\text{C}$ ]-radioactivity. Other isotope combinations (i.e. [ $^3\text{H}$ ]-radioactivity and [ $^{32}\text{P}$ ]-radioactivity) were separated empirically. It was found that from 648 to 351,584 CPM of [ $^{32}\text{P}$ ]-radioactivity the spill-over into the [ $^3\text{H}$ ]-radioactivity channel was less than 10%. The energy spectrum of [ $^{35}\text{S}$ ]-radioactivity was taken as identical to that of [ $^{14}\text{C}$ ]-radioactivity and so [ $^{14}\text{C}$ ] settings were used for [ $^{35}\text{S}$ ]-labelled samples.

## RESULTS

### 1. Optimization of the conditions for the RNA replicase assay

The assay of SFV induced RNA-dependent RNA polymerase (RNA replicase) has been described (Martin and Sonnabend, 1967; Morser, 1972) and the method used in this work is derived from their systems.

For the optimization of the reaction conditions, CEC or BHK cells were fractionated to give the "P15" cell fraction as described in Methods. This pellet was prepared from either 12 plastic Petri dishes containing confluent infected or mock-infected cells. Table 1 shows the effect of the omission of various reagents from the reaction mixture: the incorporation of [ $^3\text{H}$ ]-GTP into an acid insoluble product is independent of added template and is dependent on the presence of the 3 unlabelled nucleotides, ATP, UTP and CTP; the reaction is stimulated by the inclusion of KCl, Dextran sulphate 500 (DS500, an inhibitor of RNase activity), and an energy generating system. There is little incorporation at 0°C, and if the reaction is terminated immediately upon addition of P15 there is little or no incorporation (zero time control). The incorporation in the assay primed with a BHK mock-infected extract, in the absence of the 3 unlabelled nucleotides was higher than expected (3,932 ct/min compared with 548 in the equivalent infected assay). It is not known why this was found, but it is not thought to be significant as, in the equivalent CEC and RK assays, there was little or no radioactivity incorporated.

This assay system conforms to the specifications of Bishop (1973) that, in order to measure the net synthesis of

	CEC		RK		BHK	
	INFECTED	MOCK-INFECTED	INFECTED	MOCK-INFECTED	INFECTED	MOCK-INFECTED
Complete	19,791	237	6,725	907	314,419	2,188
- KCl & DS500	13,096	270	ND	ND	247,131	2,047
- DS500	18,640	320	ND	ND	332,141	ND
- KCl	14,325	259	ND	ND	272,975	ND
- PK & PEP	ND	ND	ND	ND	146,590	ND
- ATP, UTP & CTP	221	NS	522	204	548	3,932
Incubated at 0°C	1,242	NS	374	258	49,200	1,791
Zero time	NS	NS	NS	NS	5,237	258
+ 1µg DNase	ND	ND	ND	ND	325,433	ND

Table 1. The effect of the omission of various components from the RNA replicase assay, and the effect of the inclusion of DNase. Chick embryo cells (CEC), rabbit kidney cells (RK) and baby hamster kidney cells (BHK) were infected with SFV at a m.o.i. of 30 p.f.u./cell. The cells were harvested 6 hr after infection and fractionated to give mitochondrial pellets (P15) as described in Methods. Mock-infected cultures were treated similarly except that maintenance medium was added instead of virus. 100µl aliquots of the P15s were incubated with 250µl of assay mixture. As shown in the table above, various components were omitted, or DNase included, or the reaction immediately stopped, or the incubation was at 0°C instead of 35°C for 30 min. Assays were performed in triplicate. TCA precipitation and radioactivity determination were as described in Methods. The protein concentration of the P15s was determined. The results are expressed as specific activity of RNA replicase: ct/min/mg protein/30 min incubation, corrected for background radioactivity in the absence of any P15. ND = not done. NS = no significant radioactivity detectable above the background level.

RNA, it is necessary that the reaction mixture contains enzyme, RNA template, buffer, cations and nucleoside triphosphates. In addition a mercaptan and an energy generating system is included.

As seen in Table 1 the enzyme specific activity detected in BHK cells is greater than that detected in CEC or RK cells (and see Figure 7). Thus BHK cells were routinely used for the preparation of replicase enriched subcellular fractions. BHK cells also possess the advantage of being able to be grown in roller bottles so allowing large quantities to be prepared with comparative ease.

Martin (1969) reported that the optimum temperature for the assay of SFV replicase was 33°C. Figure 6 shows the effect of temperature on the replicase assay. Although the difference in activity between 33°C and 37°C is probably not significant, 35°C was used as the incubation temperature in all experiments.

[<sup>3</sup>H]-GTP or [<sup>3</sup>H]-UTP are widely used as the labelled substrates in RNA synthesis assays so that any contaminating polyriboadenylate polymerase or adenylylcytidylate pyrophosphorylase activity will not be detected (Eoyang and August, 1968). Actinomycin D (AMD) is included to prevent DNA-dependent RNA polymerase activity; alternatively, DNase may be included. As shown in Table 1 there is no DNA-dependent RNA polymerase activity destroyed by the inclusion of 1µg of DNase in the assay system. Table 2 shows that all 4 nucleotides may be used as the labelled substrate and that there is little, if any, homopolymerase activity present. Since a poly(C) dependent RNA replicase has been isolated from BHK cells infected with EMC (Rosenberg *et al.*, 1972) and the

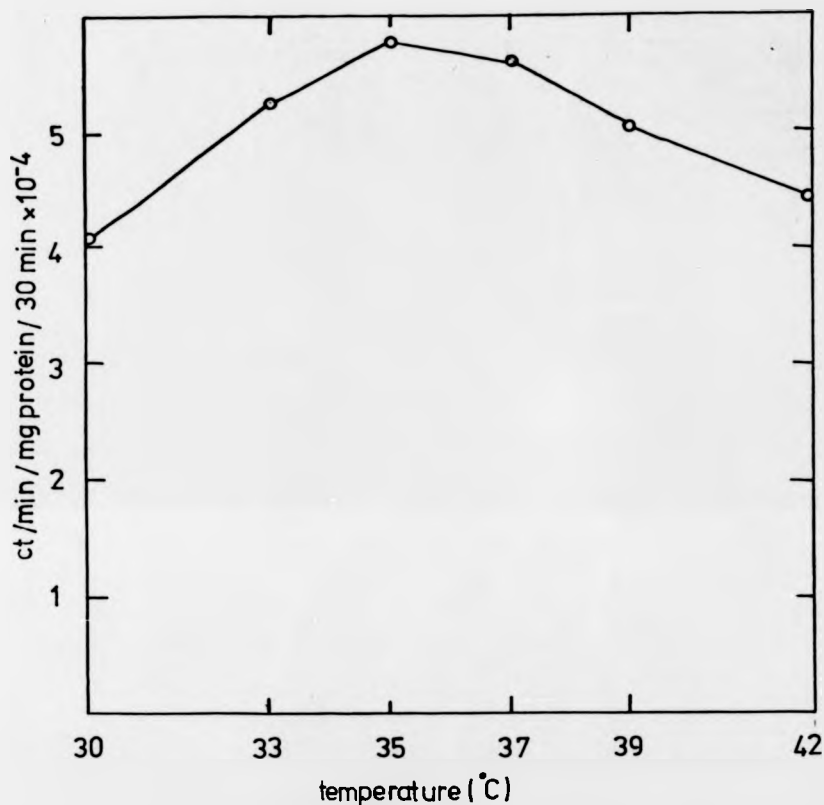


Figure 6. Optimum temperature for the assay of RNA replicase.

12 large plastic Petri dishes containing confluent monolayers of BHK cells were infected with SFV at a m.o.i. of 25 p.f.u./cell. The cells were harvested 6hr post-infection and P15 was prepared as described in Methods. 100 $\mu$ l aliquots (each containing 91 $\mu$ g of protein) were assayed for replicase activity at the temperatures shown. Results are expressed as ct/min/mg protein/30 min incubation and are the mean of triplicate assays.

+ 50nmoles of each of the                      - unlabelled nucleotides.  
3 unlabelled nucleotides.

[ <sup>3</sup> H]- ATP	11,968	614
[ <sup>3</sup> H]- CTP	22,763	1,728
[ <sup>3</sup> H]- UTP	12,150	226
[ <sup>3</sup> H]- GTP	20,149	308
( <sup>3</sup> H)- GTP) + poly C	22,428	570

Table 2. The ability of ATP, CTP, UTP and GTP to be used as the labelled substrate in the RNA replicase assay. P15, from infected BHK cells harvested 6 hr after infection, was prepared according to the Methods. 100 $\mu$ l aliquots of P15 were assayed in mixtures containing 1nmole of the labelled nucleotide (2.5 $\mu$ Ci) in the presence or absence of the three unlabelled nucleotides. The ability of the enzyme to accept poly(rC) as template was also tested: 100 $\mu$ g of poly(rC) was included with [<sup>3</sup>H]- GTP in the presence or absence of the three unlabelled nucleotides. The assays and subsequent determination of TCA-insoluble radioactivity was as described in Methods. The results are expressed as ct/min/30 min incubation. Each assay (performed in triplicate) contained the same amount of protein.



binding of the SFV replicase to poly (C) was to be investigated as a means of purifying the enzyme, the ability of the enzyme to accept poly(C) as a template in the absence of unlabelled ATP, CTP and UTP was investigated. This template was not accepted by the enzyme (Table 2), possibly because the replicase is template independent and in the presence of the normal RNA template, poly(C) is not able to compete for the enzyme. Martin and Sonnabend (1967) showed that the rate of incorporation of radioactivity was directly proportional to the amount of enzyme added, and that radioactivity was rapidly incorporated for the first 15min, thereafter the rate declined. Table 3 shows that the incorporation of [ $^3\text{H}$ ]-GTP into an acid-insoluble product was reduced by increasing amounts of cold GTP (up to 10 nmoles). The supply of [ $^3\text{H}$ ]-GTP was not exhausted after 30 min incubation as addition of further label gave no more incorporation.

## 2. Time course of RNA replicase appearance during virus multiplication

CEC or BHK cells were infected with SFV in the presence of Actinomycin D and the cells harvested at various times post-infection. (0 time post-infection (P.I.) was defined as after 1 hour virus adsorption at 37°C, at which time unadsorbed virus was removed). P15 was prepared and assayed for RNA replicase activity which is just detectable 1hr after addition of virus (0 time P.I.) with a specific activity of about 4,000 ct/min/mg protein/30 min incubation, in both chick and hamster cells. The activity detectable rises rapidly in BHK cells, plateauing at 3½ to 6hr P.I. after which time the activity falls. In chick cells the plateau was reached earlier, but the maximum

Amount of GTP in assay nmoles	Specific activity ct/min/mg protein/30min incubation
1	37,473
2	13,096
5	8,425
10	4,334
2.5 $\mu$ Ci [ $^3$ H]-GTP added for a further 15min.	38,193

Table 3. The effect of GTP concentration on [ $^3$ H]-GTP incorporation, and determination of whether [ $^3$ H]-GTP is exhausted after 30min incubation. P15 was prepared from cells harvested 6hr after virus infection, and assayed as described in Methods. The GTP concentration was varied as shown. After 30min incubation one sample, containing 1nmole of GTP, had a further 2.5 $\mu$ Ci of [ $^3$ H]-GTP added for an extra 15min incubation. Assays were performed in triplicate.

Figure 7. Time course of appearance of RNA replicase activity in infected BHK and CE cells. Confluent monolayers of either CEC or BHK cells were infected with SFV at a m.o.i. of 30 p.f.u./cell and harvested at the times indicated. P15s were prepared and assayed for replicase activity. Each time point represents the activity of replicase isolated from one large plastic Petri dish of infected cells. Results are expressed as ct/min/ $\mu$ g protein/30 min incubation and are the mean of duplicate assays.



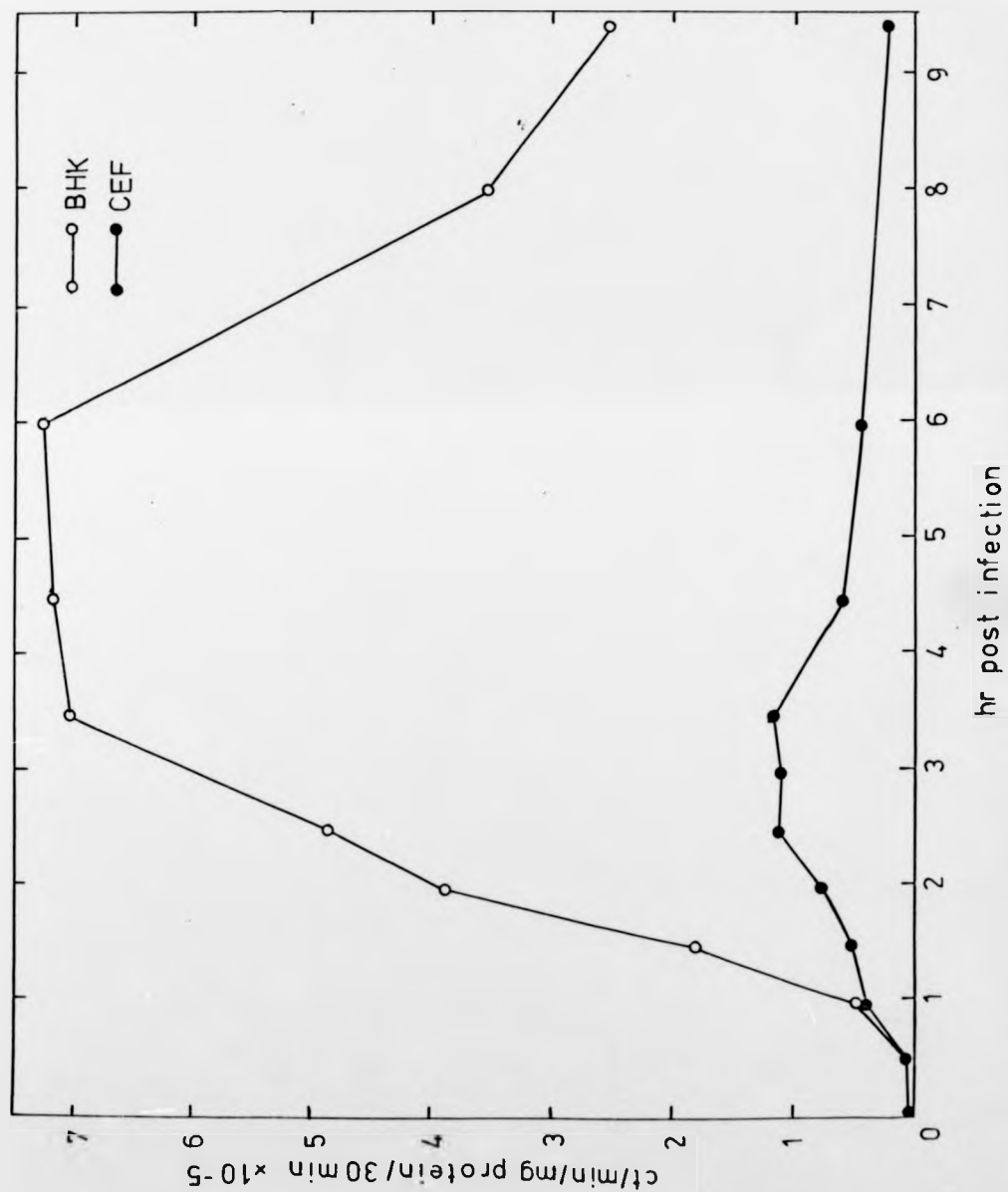
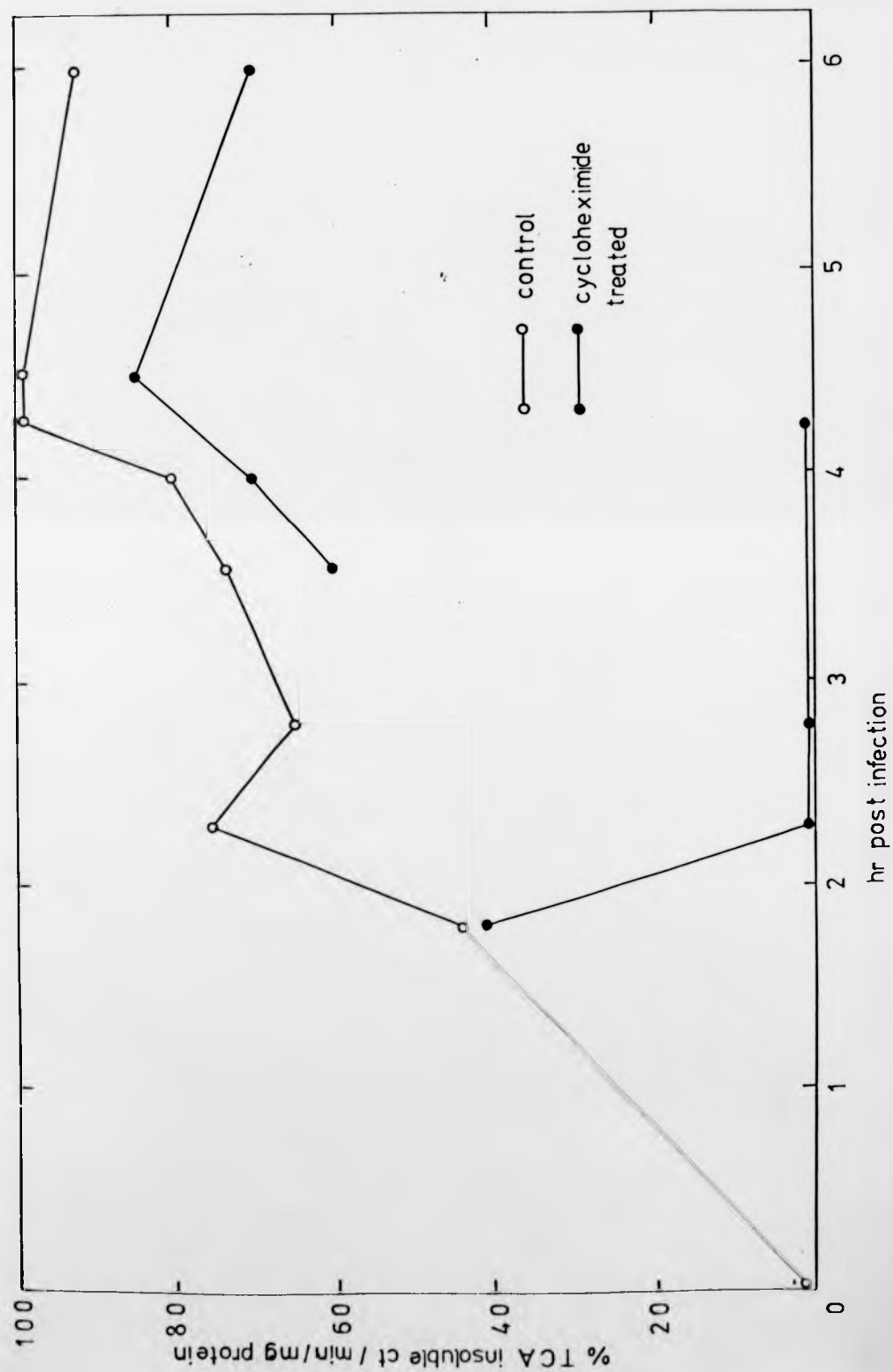


Figure 2. Effect of cycloheximide on RNA replicase activity. 34 large plastic Petri dishes containing confluent monolayers of 3T3 cells were infected with SVV at a m.o.i. of 50 p.f.u./cell. Pairs of plates untreated with cycloheximide were harvested at the times indicated (○—○). 8 plates were treated with cycloheximide at 250 µg/ml in maintenance medium, 12 hr post-infection and harvested in pairs 5, 35, 65, and 155 min later. Similarly, 8 plates were treated with cycloheximide 12 hr post-infection and harvested in pairs 5, 30, 60, and 150 min later. P15s were prepared from each pair of plates and taken up in 250 µl 0.1M NaOH. 2 100 µl aliquots were assayed for replicase activity, the remainder was used to determine protein concentration. Results are expressed as percentage specific activity of the highest determined activity (at 4 hr post-infection) Cycloheximide treated ○—○

activity detected was about one-sixth of that detected in BHK cells. (The time course in mock-infected cells was not done, it being assumed that there would be no significant activity in these cultures). Time course, Figure 7.

The effect of cycloheximide on the replicase at 1½hr and 4hr P.I. was investigated. 250µg/ml of cycloheximide was added to monolayers of BHK cells and the cultures harvested at various times later. Cultures untreated with the drug were harvested at the same times. P15, from 2 large Petri dishes, was prepared and assayed for replicase activity at each time point. Cycloheximide added at 3½hr P.I. caused an apparent rise in the subsequent levels of replicase detected (Figure 8). This is not thought to be a real effect, the results are interpreted as showing that replicase present at 3½hr P.I. is stable and does not turn over. The apparent rise is probably due to experimental variation, some difference between the pairs of Petri dish cultures. The drug added at 1½hr P.I. had a marked effect: 30 min later there was no detectable activity. To check whether the replicase, prepared at different times P.I. has different sensitivities to cycloheximide, the drug was added to the assay system containing P15 prepared from cells harvested either 1½hr or 3½hr P.I. There was no in vitro effect of the drug (Table 4). Taking the results of Friedman and Grimley (1969) and Wengler and Wengler (1975) into consideration, these results are interpreted as

Figure 8. Effect of cycloheximide on HVA replicase activity. 24 large plastic Petri dishes containing confluent monolayers of J8K cells were infected with SV at a m.o.i. of 50 p.f.u./cell. Pairs of plates untreated with cycloheximide were harvested at the times indicated (○—○). 8 plates were treated with cycloheximide at 250 $\mu$ g/ml in maintenance medium, 1 hr post-infection and harvested in pairs 5, 35, 65, and 155 min later. Similarly, 8 plates were treated with cycloheximide 3 hr post-infection and harvested in pairs 5, 30, 60, and 150 min later. P15s were prepared from each pair of plates and taken up in 250 $\mu$ l H<sub>2</sub>O. 2 100 $\mu$ l aliquots were assayed for replicase activity, the remainder was used to determine protein concentration. Results are expressed as percentage specific activity of the highest determined activity (at 4 hr post-infection.) Cycloheximide treated ○—○





Time of harvesting (hr)	Normal assay	Assay + cycloheximide
$1\frac{3}{4}$	6,329	6,174
$3\frac{1}{2}$	10,506	10,934

Table 4. Effect of cycloheximide added to the RNA replicase assay. P15 was prepared from infected cells harvested either  $1\frac{3}{4}$  or  $3\frac{1}{2}$  hr after infection. 100 $\mu$ l aliquots of the P15s were assayed for replicase in the presence or absence of 250 $\mu$ g/ml (87.5 $\mu$ g in 350 $\mu$ l total assay mixture) of cycloheximide. Results are expressed as specific activity (ct/min/mg protein/30 min incubation) and are the mean of 3 separate determinations.

showing that the replicase is synthesised before 3 $\frac{1}{2}$ hr P.I. and after this time it is stable. These ideas will be discussed more fully later.

### 3. Subcellular fractionation of infected cells

Infected monolayers of BHK cells were harvested at 4hr P.I., the cells dounced and fractionated into a nuclear fraction and post-nuclear supernatant by low speed centrifugation. The post-nuclear supernatant was then centrifuged to give a so-called "mitochondrial" pellet (PI5) and post-mitochondrial supernatant. The protein concentration of each fraction was determined and aliquots, containing approximately the same amount of protein, were assayed for replicase activity (figure 9). The highest specific activity was associated with the mitochondrial pellet (PI5). The nuclear pellet was further analysed by a second Dounce homogenization and subsequent low speed centrifugation. Most replicase activity remains associated with the pellet and is assumed to be bound to membranous structures not easily removed from the nuclei. Attempts to further fractionate the replicase by differential centrifugation did not result in release of activity from either P15 or the nuclear fractions. Usually there was loss of activity (not shown).

Since the replicase is associated with particulate structures (Friedman et al., 1972; Raul, Egly and Kempf, 1974) the use of detergents to separate it from membranes was investigated. The dounced cells were treated with either Triton N-101 (TN101), a non-ionic detergent, or a mixture of Triton N-101 and sodium deoxycholate (DOC), an anionic detergent as shown in Table 5 and then fractionated into P15 and

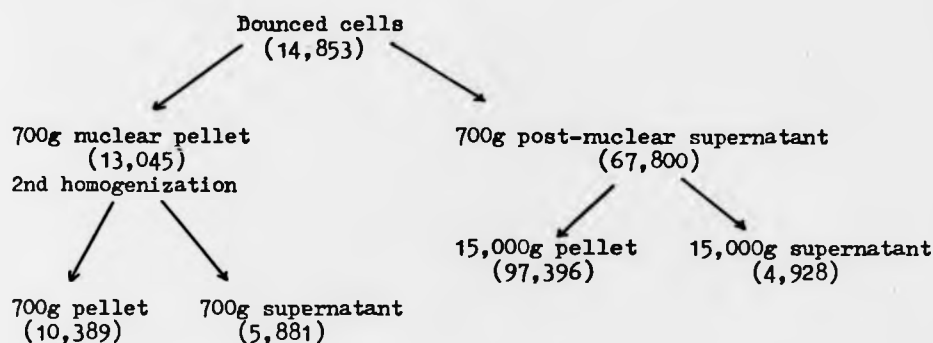


Figure 9. Fractionation of infected cells and distribution of RNA replicase activity. 18 large plastic plates of confluent monolayers of BHK cells were infected with SFV (50 p.f.u./cell) and harvested 4hr post-infection. The cells were fractionated as described in Methods except that the nuclear pellet was subjected to a further homogenization and low speed centrifugation. The protein concentration of each of the fractions was determined, and all fractions except the 15,000g diluted to 1mg/ml with RSEM. This supernatant had a protein content of 0.34 mg/ml. 100 $\mu$ l aliquots of each fraction was assayed in triplicate for replicase activity. Results, in parentheses, are specific activity of the replicase: ct/min/mg protein/30 min incubation.

Treatment	Specific activity ct/min/mg protein/30 min incubation	
	P15	S15
Untreated	156,824 (100%)	NS
Anionic + non-ionic detergents	52,517 (25%)	152,941 (75%)
Non-ionic detergent	111,785 (71%)	45,685 (29%)

Table 5. Effect of detergents on distribution of RNA replicase activity following subcellular fractionation. Confluent monolayers of BHK cells infected with SFV at a m.o.i. of 50 p.f.u. per cell were harvested at 4 hr post-infection as described in Methods. The recovered cells were homogenized and the lysate divided into 3 equal volumes: 1 containing 2% (v/v) Triton N-101, 1 containing 1% (w/v) sodium deoxycholate and 2% (v/v) Triton N-101, the third fraction was untreated. After incubation at 35°C for 3min the lysates were diluted 5-fold and fractionated into P15s and S15s as described in Methods. Replicase activity was determined. The results are expressed as ct/min/mg protein/30 min incubation. The figures in parentheses represent the percentage of recovered replicase in each fraction. NS = no significant TCA-insoluble radioactivity above the background level.

S15 and the replicase activities of each fraction determined. (The use of an anionic-non-ionic detergent mixture was based on the method of Ehrenfeld, Maizel and Summers, 1970). Triton N-101 releases only 29% of the recovered replicase activity into the supernatant. The mixture of anionic and non-ionic detergents releases 75% of the recovered replicase activity into the supernatant.

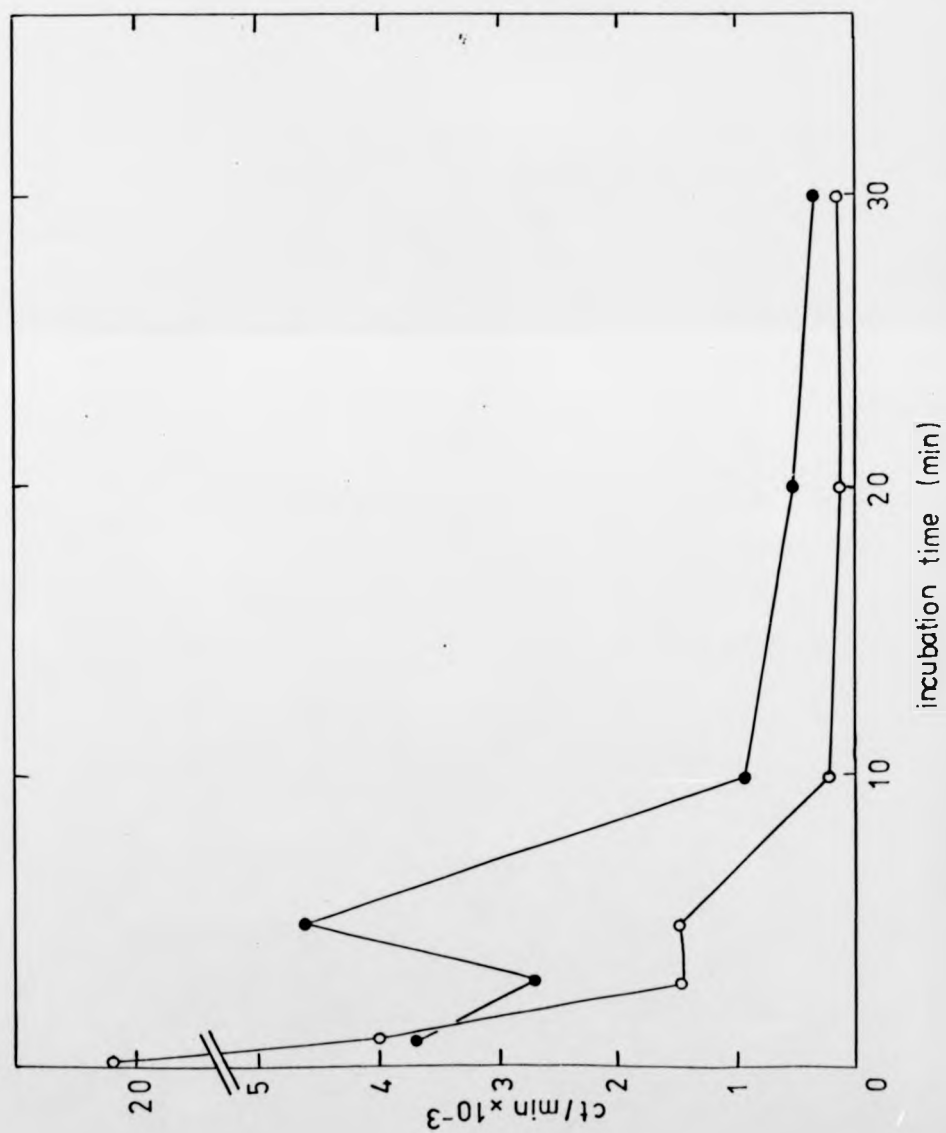
The effect of the mixture of detergents on P15 was investigated by determining the time course of release of replicase activity into the supernatant. P15 was prepared and divided into 7 equal aliquots, to 6 of these the detergent mixture was added before incubation at 35°C for the times shown in Figure 10. After centrifugation to give a second P15 and an S15 it was found that maximum release of activity from the pellet into the supernatant occurs after 5 min incubation; further incubation destroyed activity.

Thus replicase activity can be released from membranes by the use of detergents, and, if this activity were stable, the method would be a powerful one for the purification of the replicase. The S15 detergent-supernatants obtained were analysed on sucrose gradients and, in no case, was replicase activity recovered. It appears that the replicase activity released by the detergent mixture is highly labile and that unless a means of stabilizing the activity is found the method cannot be used to purify the replicase. Preliminary experiments to do this by including 50% (v/v) glycerol or by including KCl at 500mM were unsuccessful.

#### 4. Product of the invitro reaction

The RNA-synthesizing activity isolated from infected

Figure 10. 'Colubilization' of RNA replicase activity from P15.  
24 large plastic Petri dishes containing confluent monolayers  
of cells were infected with SVV at a m.o.i. of 50 p.f.u./  
cell. The P15 was prepared according to Methods, and divided  
into equal fractions to which 0.1 ml of 5% (v/v) DMSO, 10% (v/v)  
DMSO, 5-101 in RSE, was added. The fractions were incubated at  
35°C for the times indicated. They were then diluted to 3 ml with  
water and centrifuged (15,000g, 20 min at 4°C) in 3x3 ml centrifuge  
tubes. 100 µl aliquots of each pellet and supernatant were  
assayed for replicase activity. Results are expressed as 5CA  
fixable ct/dn, and are the mean of triplicate samples.  
○ — ○ supernatants, ○ — ○ pellets.



cells should be capable of synthesizing the RNA species found in the infected cell. These are 42S and 26S RNA, the major single-stranded species and the replicative intermediate (RI), a multi-stranded species. The role of the double-stranded RF is not known but is probably not involved in RNA synthesis, it may be an end product or artefact of extraction (see also in Discussion). RNA synthesis in the cell on a single-stranded template involves the production of negative strand 42S which in turn acts as template for synthesis of 42S and 26S positive strand RNAs (Bruton & Kennedy, 1975).

In order to investigate the RNA synthesized in vitro by RNA replicase preparations isolated from BHK infected cells, RNA was isolated from the reaction mixture (as described in Methods) and analysed on sucrose gradients or polyacrylamide gels. Figure 11 shows the sedimentation profile of total radiolabelled RNA, and RNase resistant RNA extracted from a reaction mixture incubated with a sample of P15. The product is RNase resistant RNA, presumably double-stranded. In order to resolve any 42S synthesized the time of centrifugation was decreased and a similar though displaced profile was obtained (not shown). The conditions of preparation of P15, and the assay conditions, were varied to see if there was any effect on RNA synthesized in vitro. The method of lysing the cells was checked: P15s prepared from cells dounced in the presence of 40% (v/v) glycerol, or from cells lysed by freezing and thawing (3 times) from  $-70^{\circ}\text{C}$  to  $20^{\circ}\text{C}$ , were only competent to synthesize double-stranded RNA (not shown). Increasing the KCl concentration to 200mM in the assay likewise had no effect on the RNA synthesized. When 31,520 ct/min of [ $^{32}\text{P}$ ] -labelled virion RNA was added to the assay in the presence of P15,



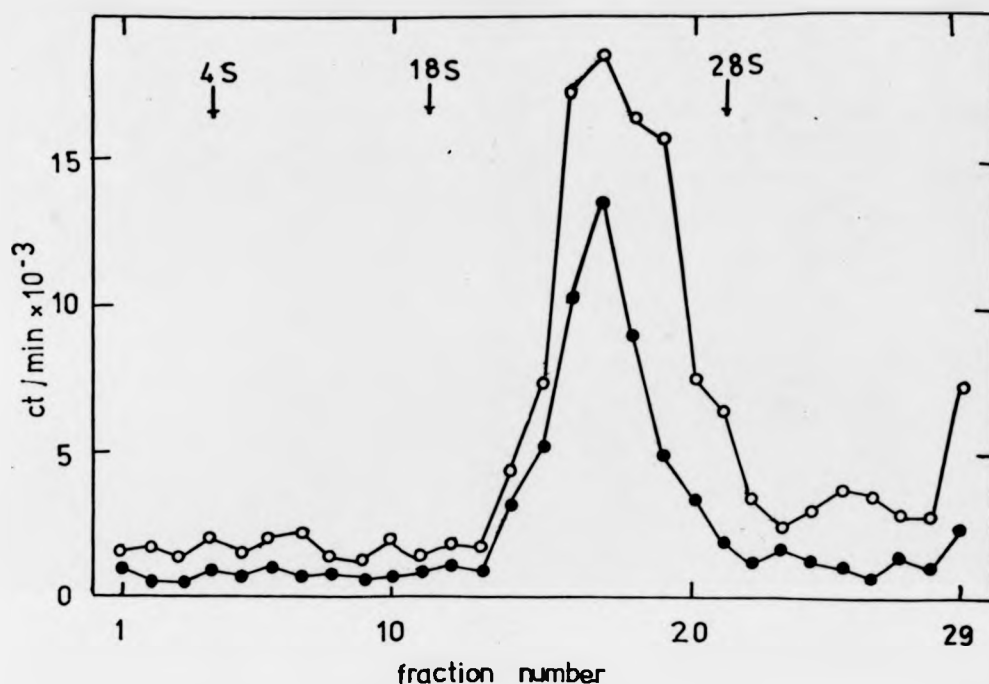


Figure 11. Sucrose gradient analysis of the replicase reaction product. P15 (at approx. 1mg/ml) was prepared from infected BHK cells and 100 $\mu$ l incubated in the standard reaction mixture for 30min. RNA was extracted with SDS-phenol as described in Methods and precipitated overnight with 2½ volumes of ethanol at -20°C. The recovered RNA was resuspended in TNE buffer and divided into 2 equal volumes. To one NDS was added to 0.1% (w/v). The other was treated with a mixture of T<sub>1</sub> and pancreatic RNases for 30 min at 37°C as described in Methods. The reaction was stopped by adding NDS to 0.1% (w/v). Analysis was by sedimentation at 135,000g for 3hr at 10°C through 4.8ml linear 6 to 30% (w/v) sucrose gradients prepared in TNE buffer containing 0.1% (w/v) SDS. Fractionation was by upward displacement into TCA. Acid insoluble radioactivity was determined.

○—○ total RNA, ●—● RNase resistant RNA. The arrows indicate the position of cellular [<sup>14</sup>C]-labelled RNA run in a parallel gradient (these markers were kindly supplied by Dr. S.I.T. Kennedy).

27,960 ct/min were recovered after TCA precipitation. As the integrity of the 42S RNA was not analysed after this incubation it may be that the RNA was digested but that only 11% of it was of a size small enough to be TCA soluble. Also, the 42S RNA may have not penetrated the replication complex where endogenous RNase was acting.

The reaction product was also analysed on 2% cylindrical polyacrylamide gels containing 0.5% agarose. Figure 12a shows the total radiolabelled RNA extracted from the reaction mixture, 12b the RNase resistant RNA. The profile shown in Figure 12c represents the RNA species extracted from infected cells. The identity of the various species are in accordance with Martin and Burke (1974). RI does not enter the gels. The material at the top of the gel (12a) is not thought to be RI as RNase treatment does not convert it to RF (12b: there is still material at the top of the gel.)

Thus as has been found (Martin and Sonnabend, 1967 ; Martin, 1969; Levin and Friedman, 1971) the replicase, after any purification step, is only competent to synthesize double-stranded RNA. The RNA synthesized by the replicase at all stages subsequent to P15 in the purification procedure was also RNase resistant.

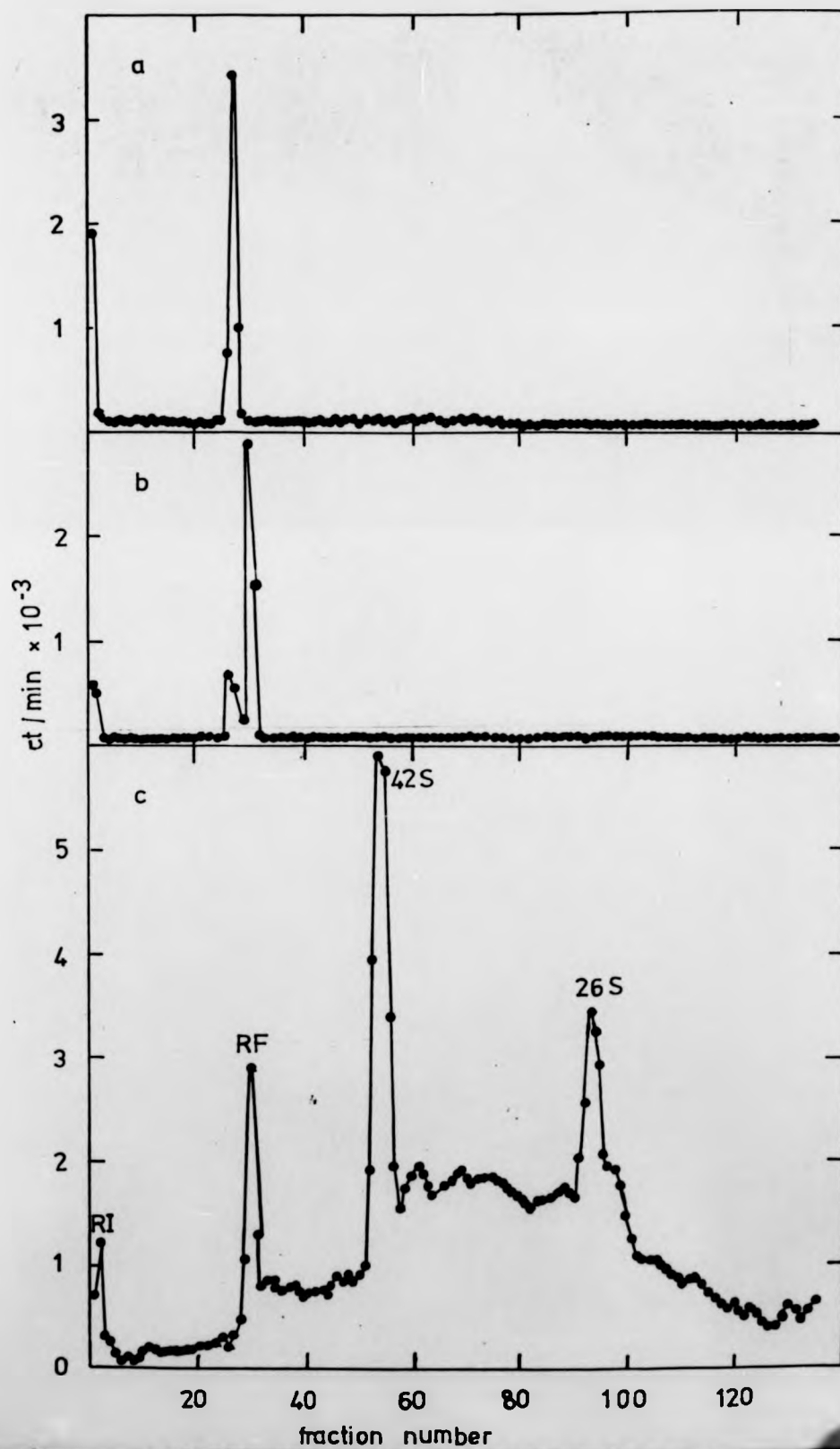
Michel and Gomatos (1973) showed that a crude fraction prepared from SFV infected BHK cells was able to synthesize double-stranded RNAs and single-stranded 42S, 26S and 22S RNA. Following their method 12 roller bottle cultures of confluent BHK cells were infected with SFV at a m.o.i. of 50 p.f.u./cell. At 4hr P.I. the maintenance medium was removed and the cells washed with ice-cold PBS containing 20 µg/ml of polyvinyl sulphate (PVS). The cells were harvested

Figure 12. Polyacrylamide gel electrophoresis of the replicase reaction product compared with the RNA species extracted from infected cells. The RNA synthesized *in vitro* was extracted and prepared as for Figure 11. For the preparation of viral specified RNA synthesized *in vivo*, infected cells were labelled with [ $^3$ H] uridine from 2 to 6hr post-infection as described in Methods. The cells were lysed by gentle homogenization in lysis buffer containing 1% (w/v) SDS. The nuclei were removed by centrifugation (800g, 10min). The supernatant was made 1-5% (v/v) in SDS and RNA extracted and recovered as described in Methods. Electrophoresis was in 20cm Perspex tube; the 2% gels were prepared and run exactly as described in Methods.

Panel a : total labelled RNA extracted from *in vitro* reaction mixture.  
 Panel b : Case resistant RNA extracted from *in vitro* reaction mixture.  
 Panel c : labelled viral RNA extracted from infected cells. The labels show the identity of the various species.

3  
2  
1  
2  
1  
5  
4  
3  
2  
1  
0

ct/min  $\times 10^{-3}$



into universal bottles, pelleted and resuspended in RSBM containing 20 µg/ml PVS. They were then lysed by Dounce homogenization. The homogenate was centrifuged for 10min at 250 xg. The supernatant (S1) was divided into 2 equal volumes, one of which was used to prepare P15. 700µl of the nuclear pellet (P1), the supernatant and P15 (all containing approximately 100µg of protein) were assayed in the reaction mixture scaled up to a total volume of 2.45ml (1.75ml of assay components plus 700µl of the cellular fraction).

Extraction of the RNA was with a mixture of SDS, Brij 58 and EDTA (see legend to Figure 13). As shown in Figure 13 P1 and S1 contained replicase activity competent to synthesize RNA which sedimented at about 26S and 42S. The P15 fraction synthesized RNA which sedimented at about 20S. Thus crude fractions contain enzyme capable of synthesizing 26S and 42S RNA, whereas a more purified fraction, analysed identically, is only able to synthesize 20S RNA. It might be argued that single-stranded synthesizing ability is lost into the 15,000g supernatant. However, ideas on the mechanism of RNA replication in which double-stranded RNA is not involved are current (Thach et al., 1974), and since no replicase activity was ever detectable in the 15,000g supernatant (see Figure 9 and Table 5) it seems likely that the double-stranded RNA synthesizing ability studied here is the true SFV replicase. It may be that some unknown factor necessary at some stage in the synthesis or release of single-strands remains in the 15,000g supernatant after centrifugation. However, addition of S15 to P15 before assaying and extracting the RNA synthesized had no effect on the sedimentation profile obtained either by the method used in Figure 11 or Figure 13a. Therefore, either

Figure 13. Sucrose gradient analysis of the replicase reaction products from 3 different subcellular fractions.

P1, S1 and P15 were prepared and incubated in the replicase reaction mixture as described in the text. After 30min incubation at 35°C S1 and P15 reaction mixtures were made to 20mM EDTA, 1.95% (w/v) SDS and 0.5% (w/v) Brij 58. The P1 mixture was centrifuged at 250g for 10min to yield a supernatant which was similarly treated with EDTA-SDS-Brij 58. Analysis of the detergent treated reaction mixtures was by sedimentation at 76,000g for 16hr at 10°C through 12ml linear 15 to 30% (w/v) sucrose gradients prepared in TNE buffer containing 0.1% SDS.

Fractionation was by upward displacement, samples were collected into Triton-toluene scintillation and radioactivity determined. Before the addition of the EDTA-SDS-Brij mixture samples of P15 and S1 were RNase treated as described in Methods.

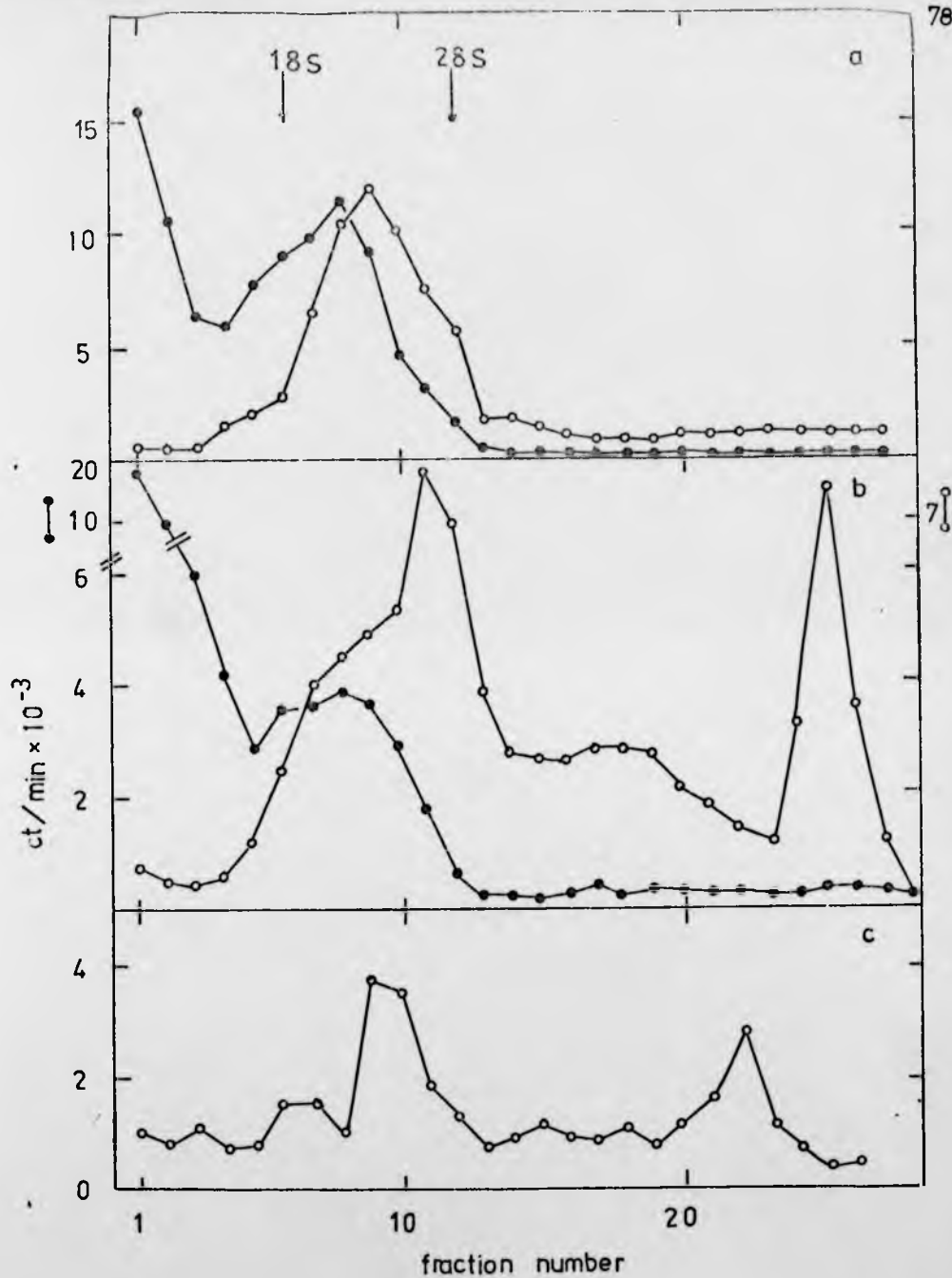
Panel a: P15 product. o—o Total RNA ●—● RNase treated

Panel b: S1 product. o—o " " ●—● " "

Panel c: P1 product. o—o " "

The arrows indicate the position of cellular [<sup>14</sup>C]-labelled RNA markers run in a parallel gradient.

ct/min × 10<sup>-3</sup>



single-stranded RNA is not released and the extracted product, after deproteinization, becomes double-stranded (see Weissmann, Feix and Slor, 1968), or disruption of the replication complex leads to endogenous RNase destroying the product RNA. To investigate whether there is any single-stranded RNA present in the in vitro reaction, but is not released, the effect of RNase on the replicase assay was determined.

#### 5. Effects of RNase on the replicase

If either 1 or 20 $\mu$ g of RNase was added after 30min incubation in the assay mixture, and the incubation continued for a further 30min, there was no reduction in the TCA precipitable counts recovered (Figure 14, closed circles). The product, after 60min incubation, must, therefore, be wholly double-stranded. If RNase (from 1 to 50 $\mu$ g) was added to the assay, and was present during the incubation, 40 to 50% of the TCA insoluble counts detected in the absence of RNase were recovered (Figure 14, open circles). Indeed, 1 $\mu$ g of RNase was sufficient to reduce incorporation by 50%. Therefore, during the synthesis of the double-stranded product there is a single-stranded stage. If the enzyme preparation was preincubated for 30min at 37°C with either no RNase or 1 and 20 $\mu$ g of RNase there was no significant difference between the TCA precipitable counts detected (Figure 14, closed squares). The amount of [ $^3$ H]-GTP incorporation was, however, 75% less than that detected using P15 which was not preincubated. In the presence or absence of RNase the template is single-stranded and is destroyed during preincubation.

The conclusion that the template for the replicase is 75% single-stranded, that the final product is double-stranded and



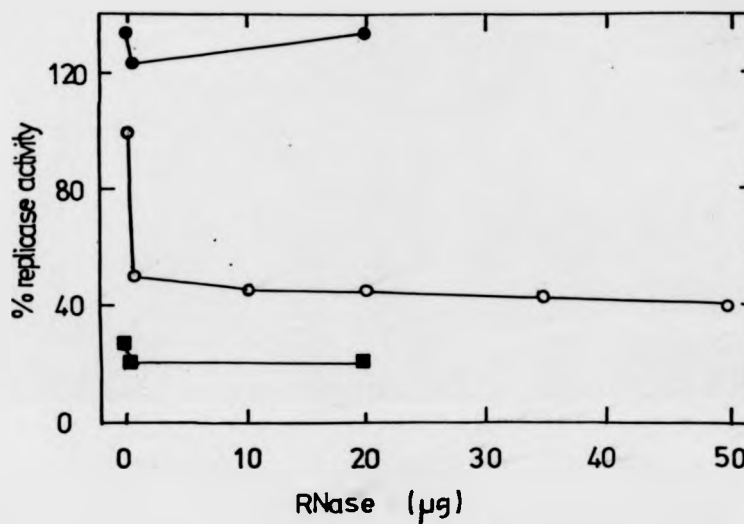


Figure 14. Effect of RNase on the TCA insoluble counts recovered from the replicase assay. P15, at 1mg/ml of protein, was prepared as described. 100μl aliquots were assayed for replicase activity under the conditions given below. Pancreatic RNase was kept as a 5mg/ml stock in SSC buffer.

●—● RNase (at the concentrations shown) was added to the assay containing P15 after 30min incubation and then incubated for a further 30min before the reaction was stopped by adding TCA and determining TCA insoluble counts incorporated as described in Methods.

○—○ RNase during the 30min assay.

■—■ 100μl P15 incubated with RNase (30min, 37°C), then 250μl assay mixture added and the whole incubated for a further 30min before determining TCA insoluble counts.

Results are expressed as percentage of the ct/min determined in the standard assay without RNase.

that single-stranded RNA is synthesized during the course of the assay, but is not released from the template complex, is supported by the observation that the time course of the reaction (Figure 15) shows similar kinetics in the presence or absence of RNase. However, the amount of RNA synthesized was less in the presence of RNase as any single-stranded RNA would have been digested and the only RNA recovered would have been that part of the RI which was double-stranded. Figure 15 also supports the idea that it is the single-stranded template that is destroyed during preincubation since the RNA synthesized after 60min incubation is 68% of the amount synthesized after 30min, rather than 25% (compared with the sample not preincubated) which is synthesized after a 30min assay following a 30min preincubation (Figure 14, closed squares).

#### 6. Characterization of the replication complex on isopycnic sucrose gradients

Using the technique of isopycnic centrifugation to fractionate a cytoplasmic extract of chick cells infected with SFV, Friedman *et al* (1972) found one band at 37% (w/w) sucrose which was enriched with cytopathic vacuoles (CPV-1), viral RNA species and RNA replicase. Figure 16 shows the redistribution of membrane fractions, on an isopycnic gradient, from BHK cells infected with SFV and harvested 4hr P.I. Characteristically, two main bands were seen on gradients loaded with P15 from mock-infected cells whereas after virus infection there were more visible bands. If a gradient obtained from infected cells was fractionated and 100 $\mu$ l aliquots of the fractions assayed for RNA replicase activity, then replicase was routinely found in a band of about 35% (w/w)

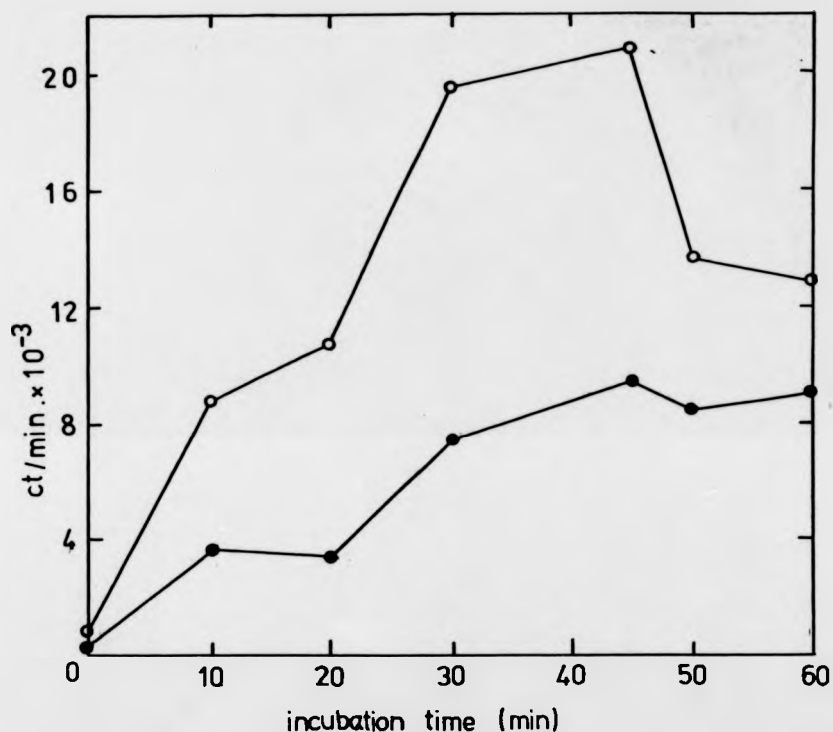


Figure 15. Time course of the replicase reaction in the presence and absence of RNase. P15 was prepared (at 1mg/ml of protein) and 200 $\mu$ l aliquots added to two separate assay mixtures scaled up to a final volume (containing P15) of 700 $\mu$ l. One contained 25 $\mu$ g pancreatic RNase ( $\bullet$ — $\bullet$ ), the other was the untreated control ( $\circ$ — $\circ$ ). At the times indicated 100 $\mu$ l aliquots were removed into TCA and the insoluble counts determined.

## MOCK-INFECTED



## INFECTED



Figure 16. Membrane bands prepared from infected or mock-infected cells. BHK cells from 10 roller bottle cultures were used as the source of either infected or mock-infected P15s. This pellet was resuspended in 5ml 30% (w/w) sucrose in RSBM buffer. This was layered onto 3ml of 65%, 4ml of 45%, and 4ml of 40% sucrose (all w/w in RSBM). 4ml of 25% (w/w) sucrose in RSBM and 3ml of RSBM were then layered on the sample. Analysis was by centrifugation at 96,000g for 17hr at 4° C.

sucrose (Figure 17). The incorporation of [ $^3\text{H}$ ]-uridine into viral specific RNA at different times after infection shows that there is a close association of replicase activity with the in vivo labelled RNA (Figure 18). Label was present immediately before the cells were harvested from either  $1\frac{1}{2}$  to  $1\frac{3}{4}$ ,  $3\frac{1}{2}$  to 4, or  $5\frac{1}{2}$  to 6hr P.I. DNA-dependent RNA transcription was prevented by including  $1\mu\text{g/ml}$  Actinomycin D in the medium for  $5\frac{1}{2}$ hr before the addition of the label. P15s from cells harvested at 4hr or 6hr P.I. gave similar profiles on isopycnic gradients: the replicase activity and [ $^3\text{H}$ ]-uridine TCA precipitable counts were coincident. Fractionation of P15 from cells harvested  $1\frac{3}{4}$ hr P.I. on an isopycnic gradient also showed coincidence of the two parameters, but they were further down the gradient, possibly because viral induced membrane alteration is not complete by this time. In a similar experiment, fractions 10 to 15 from an isopycnic gradient (see Figure 17) loaded with P15<sub>from</sub> cells labelled with [ $^3\text{H}$ ]-uridine  $3\frac{1}{2}$  to 4hr P.I., were pooled and the RNA extracted. This RNA was compared with RNA isolated from the nuclear pellet obtained after douncing the cells and centrifugation at 700g for 10min. As shown in Figure 19a, the labelled RNA, associated with the replicase fraction, from the isopycnic gradient was single-stranded, sedimenting at about 26S. The labelled RNA associated with the nuclear fraction was mainly RNase-resistant, sedimenting at about 20S (Figure 19b). The 26S RNA is the message for the structural proteins of the virus (Clegg and Kennedy, 1974b) and its association with fractions rich in replicase activity suggests that it is synthesized at CPV-1. There is a small amount of 42S RNA associated with the nuclear pellet, but the major portion would be associated with the nucleocapsids at CPV-2 (Friedman et al., 1972). These authors

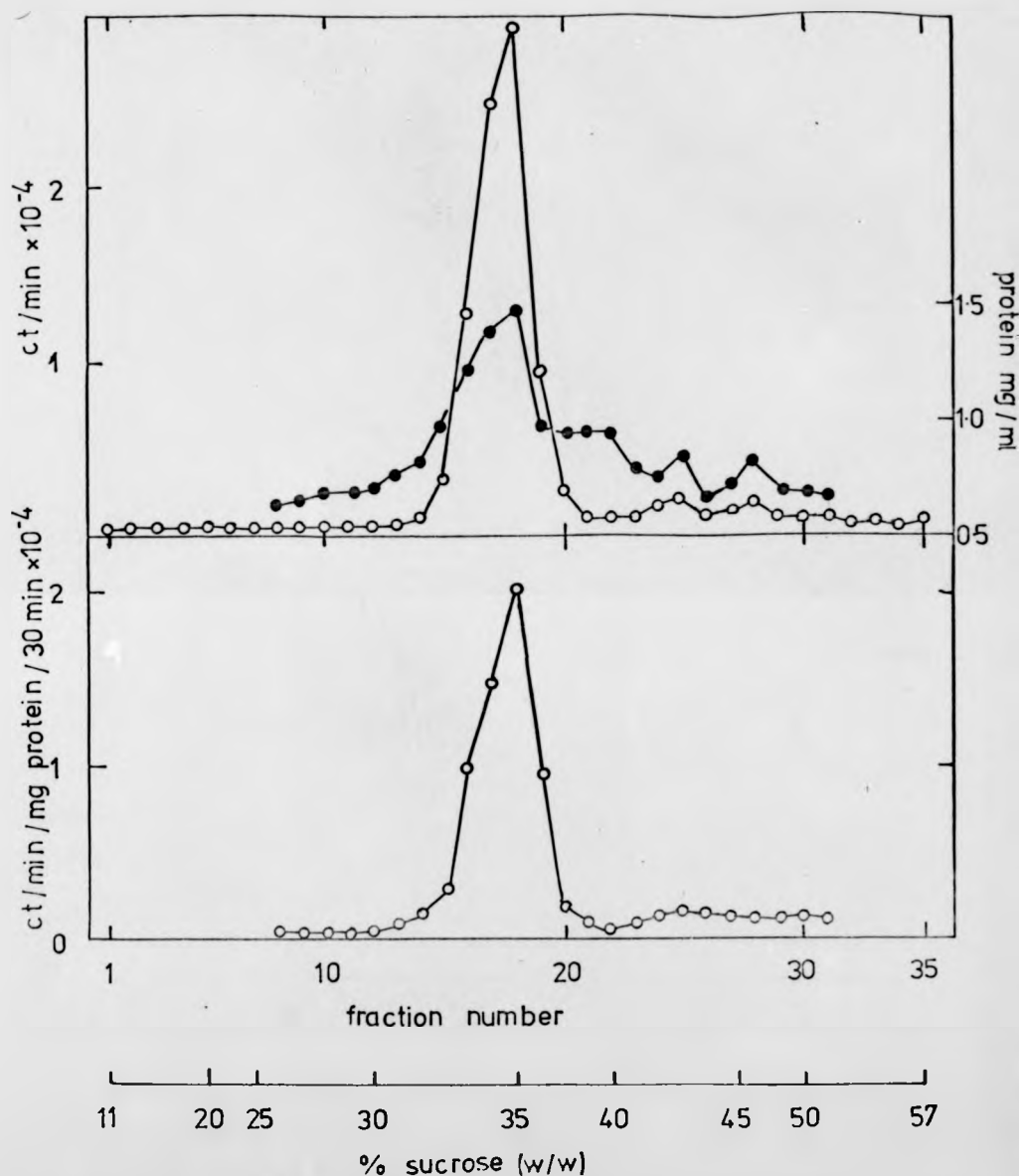


Figure 17. Distribution of replicase activity over an isopycnic gradient. P15 was prepared from the BHK cells from 10 roller bottle cultures infected with SFV. Fractionation was by isopycnic gradient centrifugation as described in the legend to Figure 16 and in Methods. The gradient was unloaded by upward displacement, and aliquots of each fraction assayed for replicase activity. The protein concentration of fractions 8 to 31 was determined. Panel a :  $\circ$ — $\circ$  TCA insoluble ct/min in vitro.  $\bullet$ — $\bullet$  protein concentration. Panel b : TCA insoluble ct/min corrected for protein conc. to give replicase specific activity: ct/min/mg protein/30 min incubation.

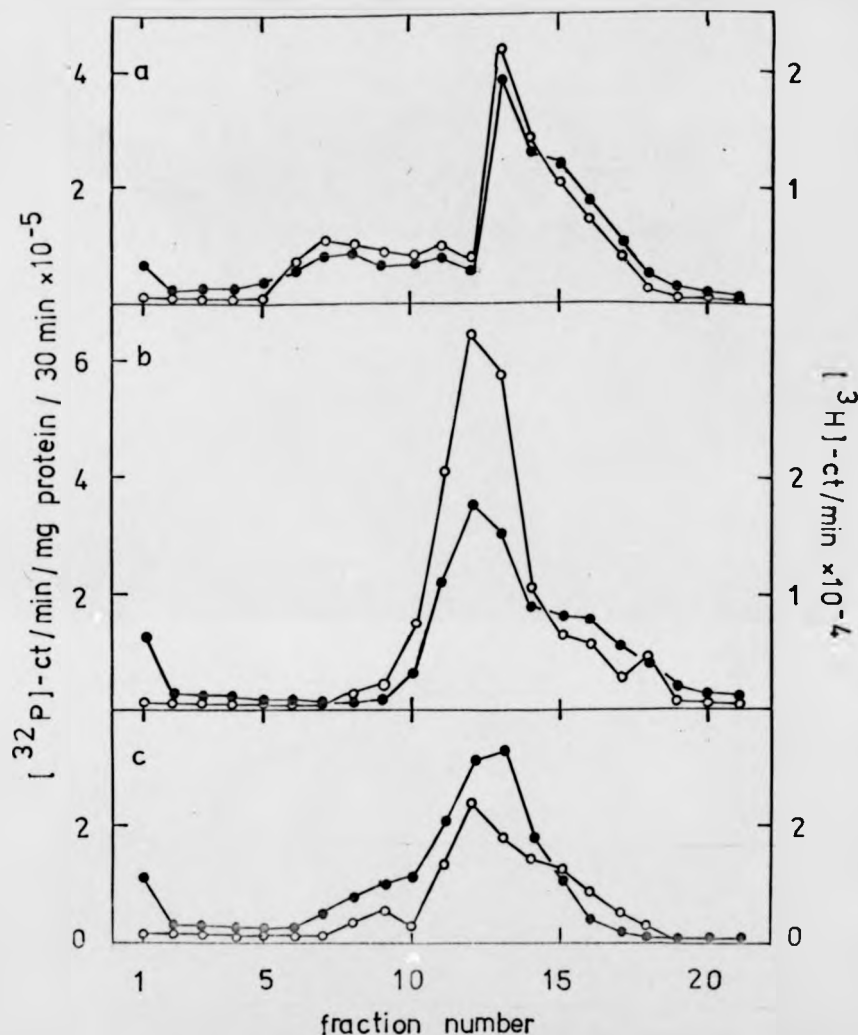


Figure 18. Coincidence of in vivo labelled viral RNA with in vitro replicase activity. For in vivo labelling cells were treated with Actinomycin D for 5½ hr before the addition of 500µCi of [<sup>3</sup>H]-uridine in EDA medium to each of two roller bottles containing confluent BHK cells. Virus infection was as described in Methods. The labelled cells were combined with unlabelled, infected cells from 4 roller bottles harvested at the same time after infection. P15s were analysed by isopycnic gradient centrifugation. The gradients were unloaded by aspiration from the top, and 100µl aliquots assayed for RNA replicase activity with [<sup>32</sup>P]-GTP as the label. The [<sup>3</sup>H]-ct/min was corrected for a 10% spill-over of the [<sup>32</sup>P]-ct/min.

Panel a : Label present 1½ to 1¾ hr post-infection, harvested at 1¾ hr.

Panel b : Label present 3½ to 4 hr post-infection, harvested at 4 hr.

Panel c : Label present 5½ to 6 hr post-infection, harvested at 6 hr.

○—○ [<sup>32</sup>P]-ct/min, replicase activity; ●—● [<sup>3</sup>H]-ct/min, in vivo labelled viral RNA.

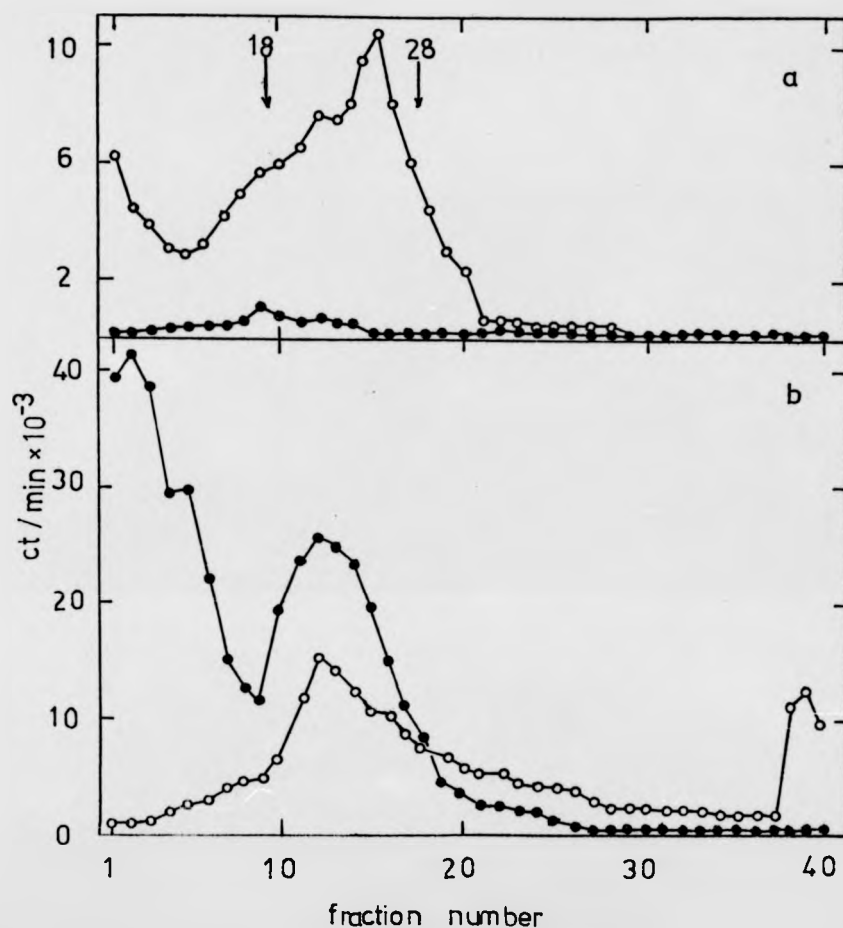


Figure 19. Viral RNA associated with 2 subcellular fractions. Infected cells were labelled with [<sup>3</sup>H]-uridine from 3½ to 4hr P.I. as described in Figure 18. Panel a: Fractions 10 to 15, enriched with replicase activity, from an isopycnic gradient were pooled and the RNA extracted as described in Methods. The recovered RNA was resuspended in TNE buffer and divided into 2 equal volumes. To one NDS was added to 0.1% (w/v). The other was treated with a mixture of T<sub>1</sub> and pancreatic RNases as described in Methods. The digestion was stopped by adding NDS to 0.1%. Analysis was by sedimentation at 135,000g for 2½hr at 10°C through 4.3ml linear 6 to 30% (w/v) sucrose gradients prepared in TNES. Fractionation was by aspiration from the top. Fractions were collected into TCA and insoluble ct/min determined: ○—○ total RNA, ●—● RNase treated RNA. The arrows indicate the position of cellular RNA markers run in a parallel gradient. Panel b: Viral RNA associated with the 700g nuclear fraction was extracted as described in Methods and analysed as above. The gradients were fractionated by upward displacement into Triton-toluene scintillant. ○—○ total RNA, ●—● RNase treated RNA



also showed that the RNA labelled from 5 to 6hr P.I., and associated with CPV-1, were the 26S, 42S and RF species.

If the infected cells were pulsed with label (1min, 5min or 30min) before harvesting at 4hr P.I., the distribution of labelled RNA over the isopycnic gradient showed little difference for each pulse time (Figure 20). Thus the site of RNA synthesis appears to be the "35%" fraction.

If cells were infected with SFV containing [ $^3\text{H}$ ]-labelled 42S RNA, 1½hr after infection the viral labelled RNA was associated with those fractions from the isopycnic gradient having replicase activity (Figure 21). This is consistent with the observations of Friedman and Sreevalsan (1970) that, 1hr after infection, input virion RNA is associated with a membrane bound replication complex. The RNA is detached later in infection (Sreevalsan, 1970) and is converted to multistranded RNA (Friedman, Levy and Carter, 1966).

When an identical amount of virus labelled with  $2 \times 10^6$  ct/min of [ $^{35}\text{S}$ ]-methionine was used to infect cell no [ $^{35}\text{S}$ ]-activity was detectable on isopycnic gradients primed with P15 prepared either 1½ or 4hr P.I. Thus the input viral structural proteins are not involved in replicase activity.

#### 7. Recovery of the replicase enriched band from isopycnic gradients

The band from the isopycnic gradient having replicase activity was recovered by diluting the sucrose with RSBM and pelleting at 50,000g for 1hr at 4°C. Further purification of this band was attempted by first treating it with detergents. The effect of several treatments on the in vitro activity is shown in Table 6. Triton N-101 had no effect on replicase activity; sodium deoxycholate (DOC) or a mixture of DOC and Triton N-101 destroyed replicase activity; a

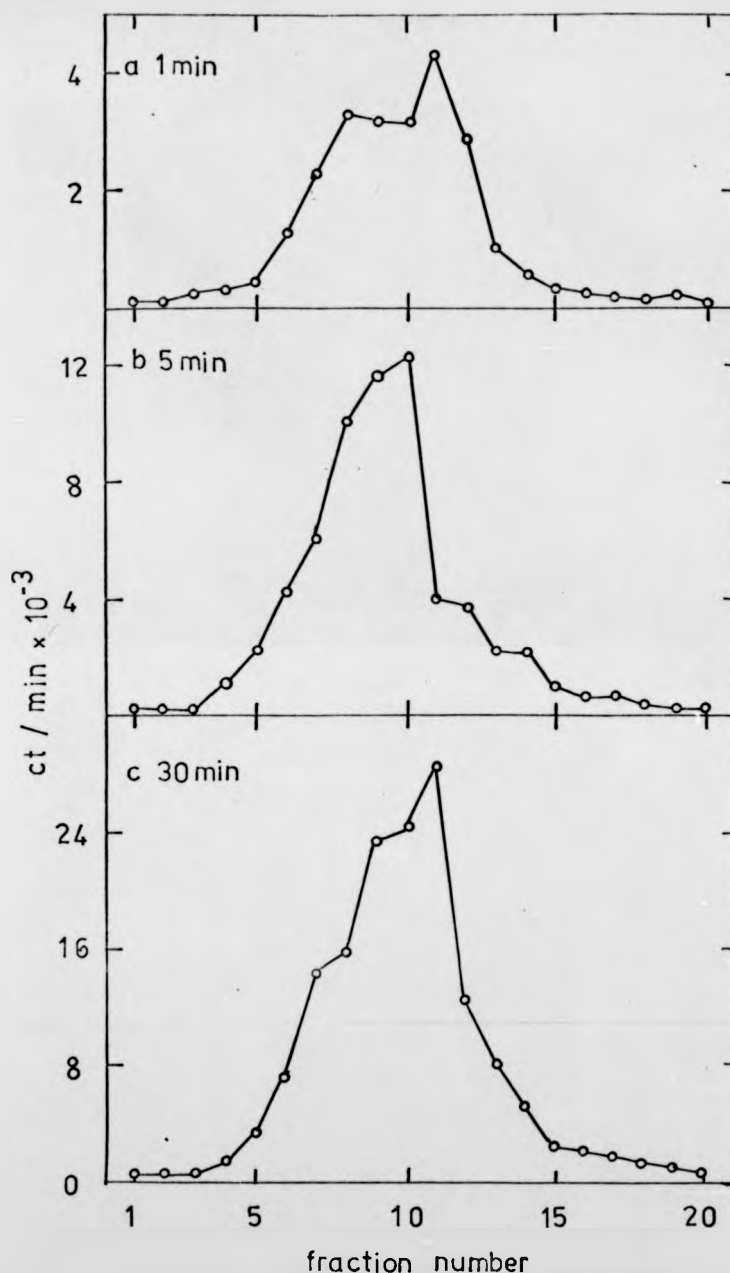


Figure 20. Distribution of pulse labelled viral RNA over isopycnic gradients. Cells were infected and labelled with [<sup>3</sup>H]-uridine as described in Figure 18 except that label was present before harvesting at 4hr post-infection for 1min, 5min or 30min. Incorporation of label was stopped by immersing the labelled roller bottles of infected BHK cells in an ice-water slurry and then washing 4 times with ice-cold PBS containing 10mM uridine. P15s were prepared and fractionated on isopycnic gradients as described in Methods. The gradients were fractionated by aspiration from the top directly into Triton-toluene scintillant and radioactivity determined.

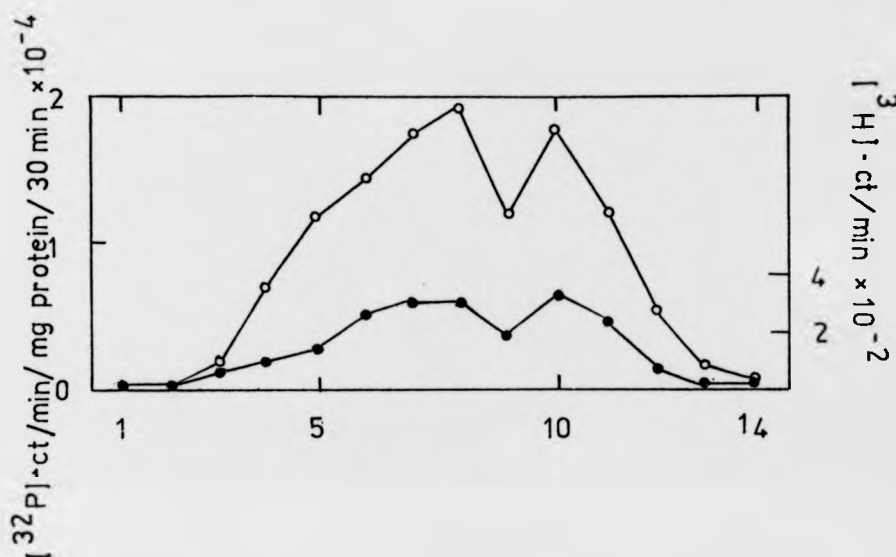


Figure 21. Association of replicase activity and input virion RNA on an isopycnic gradient. SFV containing virion RNA labelled with [<sup>3</sup>H]-uridine was prepared as described in Methods. Infection was at 50 p.f.u./cell and approximately  $5 \times 10^5$  ct/min were added to each of 6 roller bottles containing confluent BHK cells. The bottles were harvested at 12 hr P.I., P15 prepared and fractionated on an isopycnic gradient. 100  $\mu$ l aliquots of each fraction were assayed for replicase activity using [ $\alpha$ -<sup>32</sup>P]-GTP.

○—○ TCA insoluble [<sup>32</sup>P]-ct/min/mg protein/30min incubation.

●—● TCA insoluble [<sup>3</sup>H]-ct/min.

Specific activity  
ct/min/mg protein/30min incubation

active band	18,080 (100%)
+ 1% Triton N-101	19,733 (109%)
+ 0.5% DOC	282 (1.6%)
+ 0.5% DOC, 1% T N-101	1,743 (9.6%)
+ 0.5% Nonidet NP40	10,203 (57%)
+1mg/ml DS500, 0.5% DOC	16,820 (93%)

Table 6. Effect of detergents on replicase fraction isolated from isopycnic gradient. The replicase enriched band from an isopycnic sucrose gradient, loaded with P15 prepared from 12 infected roller bottle cultures of BHK cells, was recovered by centrifugation (50,000g for 1hr at 4°C). The pellet was taken up in 700 $\mu$ l of RSEM, the protein concentration of an aliquot determined, and it was then divided into 6 100 $\mu$ l volumes. These were treated with stock solutions of detergents in RSEM to give the final concentrations shown above. The detergents were added dropwise (and separately, in the case of mixtures) to give a final volume of 300 $\mu$ l. The replicase activity of 100 $\mu$ l aliquots was determined. Results are the mean of 3 assays.

mixture of Dextran Sulphate 500 (DS500) and DOC reduces activity by about 7% (detergent method or Arlinghaus and Poltanick, 1969a); Nonidet P40 reduces activity by about 43%. In a subsequent experiment DS500 was added dropwise to 1mg/ml, and DOC to 0.5% (w/v) to the pelleted replicase enriched band from an isopycnic gradient (loaded with P15 from 8 roller bottle cultures of BHK cells). This was then centrifuged at 15,000g for 30min at 4°C in a 3 X 3 ml swing-out rotor. Aliquots of the pellet and supernatant were both assayed for replicase activity (pellet : 6,350 ct/min/mg protein/30 min incubation; supernatant : 16,820 ct/min/mg protein/30min incubation). The supernatant was layered onto a linear 5 to 20% (w/v) 12ml sucrose gradient prepared in RSBM, and centrifuged for 16hr at 50,000g. There was no replicase activity detectable in any of the gradient fractions. Other conditions of centrifugation (75min at 100,000g) gave a similar result so DS500 and DOC treatment was abandoned as a means of solubilization. As will be described in the next section, replicase treated with Triton N-101 can be analysed on sucrose gradients.

#### 8. Sedimentation of replicase band from isopycnic gradients on linear sucrose density gradients

It was finally found that the recovered replicase band could be analysed on linear 5 to 65% (w/v) sucrose gradients. This was possible because of the association of replicase with RNA synthesized in vivo and RNA synthesized in vitro which is not lost (as single-strands) from the replication complex. Initially, replicase recovery was achieved by assaying the enzyme in vitro and layering the complete reaction mixture onto the gradient (Figure 22). The sedimentation of the labelled RNA differed, depending on whether 1% (v/v) Triton N-101 was

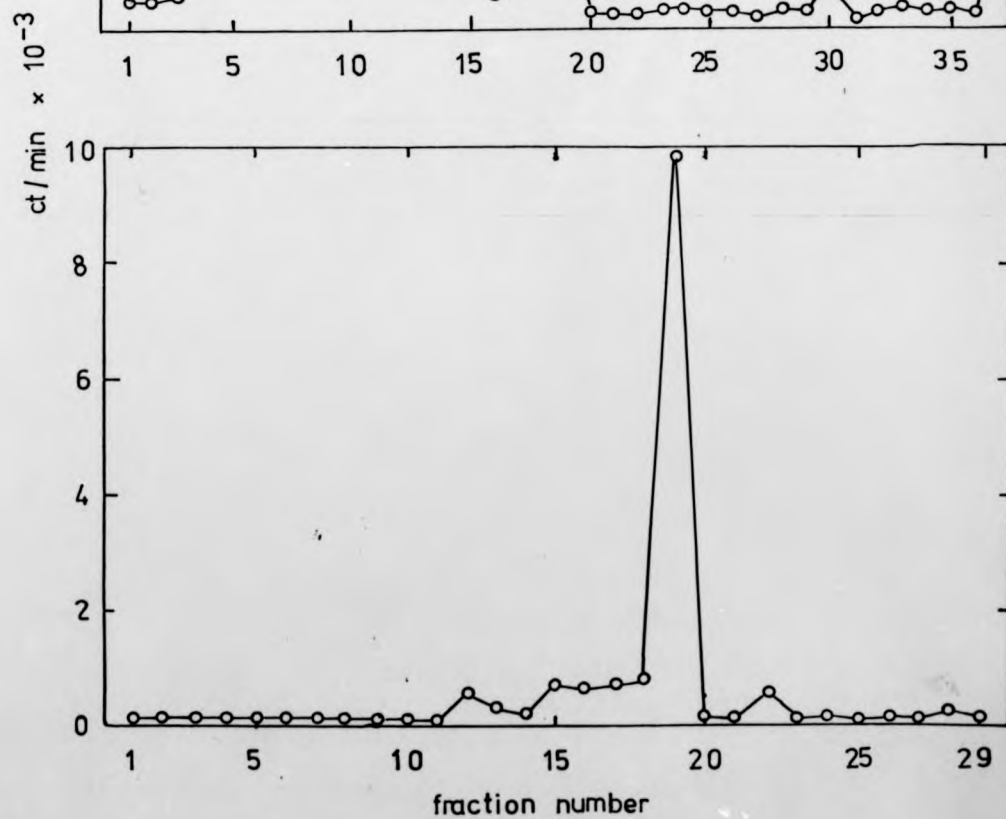
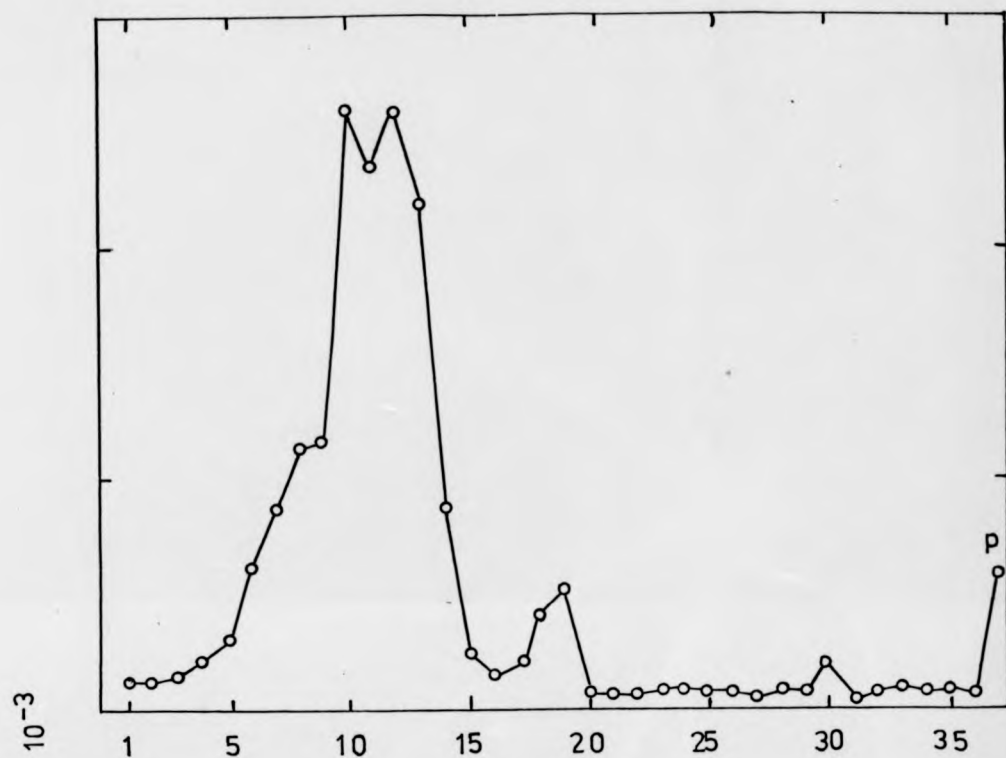
Figure 22. Sedimentation of label incorporated in replicase reaction mixture in linear sucrose gradients.

The replicase enriched fraction from an isopycnic gradient loaded with P15 from 10 roller bottle cultures of infected BHK cells was recovered by centrifugation, and the pellet resuspended in 200  $\mu$ l RSEM. This was divided into two equal volumes: to one 100  $\mu$ l of RSEM was added, to the other 100  $\mu$ l of RSEM containing 2% (v/v) Triton N-101. These fractions were assayed in a final reaction mixture volume of 700  $\mu$ l and were then layered on 12ml linear 5 to 65% (w/v) sucrose gradients prepared in RSEM. The gradient on which the Triton treated material was analysed contained 1% (v/v) Triton N-101. Analysis was by centrifugation at 200,000g for 3hr 30min at 4°C. Fractionation was by aspiration from the top into TCA. Insoluble counts were determined.

Top panel : detergent treated material on gradient containing detergent. p represents the count in the pellet.

Bottom panel : untreated with detergent.

ct / min  $\times 10^{-3}$



present in the gradient or not. The recovered label from the gradient not containing detergent was coincident with a visible band. There was a pellet present in the gradient containing detergent, in the experiment shown in Figure 22 this pellet had some associated count. There is coincidence of the in vitro labelled RNA with in vivo labelled RNA on these gradients (Figures 23 and 24). Two totally independent experiments are shown in Figure 24, in one (bottom panel) the pellet had associated in vitro and in vivo label. In the experiment shown in the top panel, the recovered band from the isopycnic gradient was resuspended much more thoroughly in detergent so achieving complete solubilization.

The in vitro and in vivo peaks of labelled RNA were in the fraction associated with replicase activity (Figure 25). Recovered material from the isopycnic gradient was analysed on linear gradients and shown to have replicase activity by assaying fractions from the linear gradient. Material not treated with detergent sedimented at about 250S, material treated with detergent at about 25S (each value is an average of 4 determinations). In Figure 25b the spin time was increased to 4hr 25min to check if the S-value changed during a longer spin.

Occasionally it was found that there was no pellet present in the detergent containing gradients, instead there was a visible band near the bottom of the tube (Figure 26). This had an S-value of about 260. Thus it appears that detergent treatment of the replicase enriched fraction from the isopycnic gradient converts the replication complex from 250S to 25S. The replication complex is associated with RNA made in the infected cell, product RNA of the replicase in vitro which is not released as single-strands, and replicase activity.



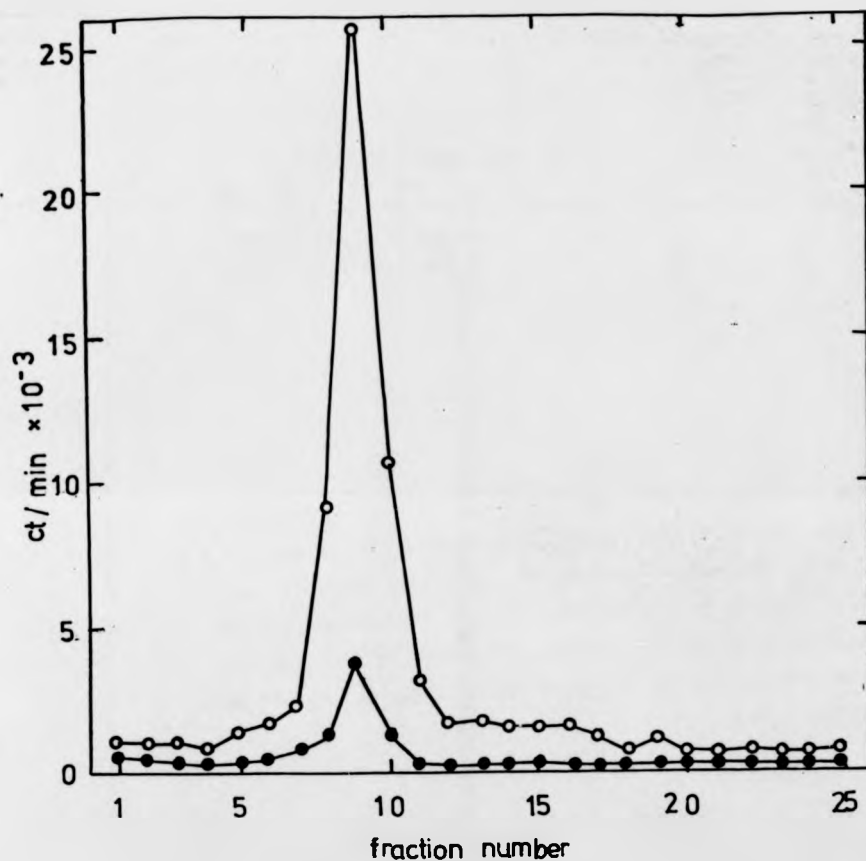


Figure 23. Coincidence of in vivo labelled RNA with in vitro synthesized RNA on a linear sucrose gradient in the absence of detergent. The procedure was the same as for Figure 22 in the absence of detergent except that two of the roller bottle cultures were each incubated with 500 $\mu$ Ci [<sup>3</sup>H]-uridine for  $\frac{1}{2}$ hr before harvesting at 4hr post-infection. [<sup>32</sup>P]-GTP was the in vitro label

○—○ [<sup>32</sup>P]ct/min.  
 ●—● [<sup>3</sup>H]ct/min.

Figure 24. Coincidence of *in vivo* labelled RNA with *in vitro* synthesized RNA on linear sucrose gradients in the presence of Triton N-101. The procedure was the same as Figure 22 in the presence of detergent except that two of the roller bottle cultures were each inoculated with 500  $\mu$ Ci of [ $^3$ H]-uridine for 4 hr before harvesting at the post-infection. [ $\alpha$ - $^{32}$ P]-GTP was the *in vitro* label.

○ — [ $^{32}$ P]ct/min.  
 ● — [ $^3$ H]ct/min.

Two separate experiments are shown, in one (bottom panel) the pellet (p) had associated TCA insoluble count, in the other (top panel) there was no count associated with the pellet.

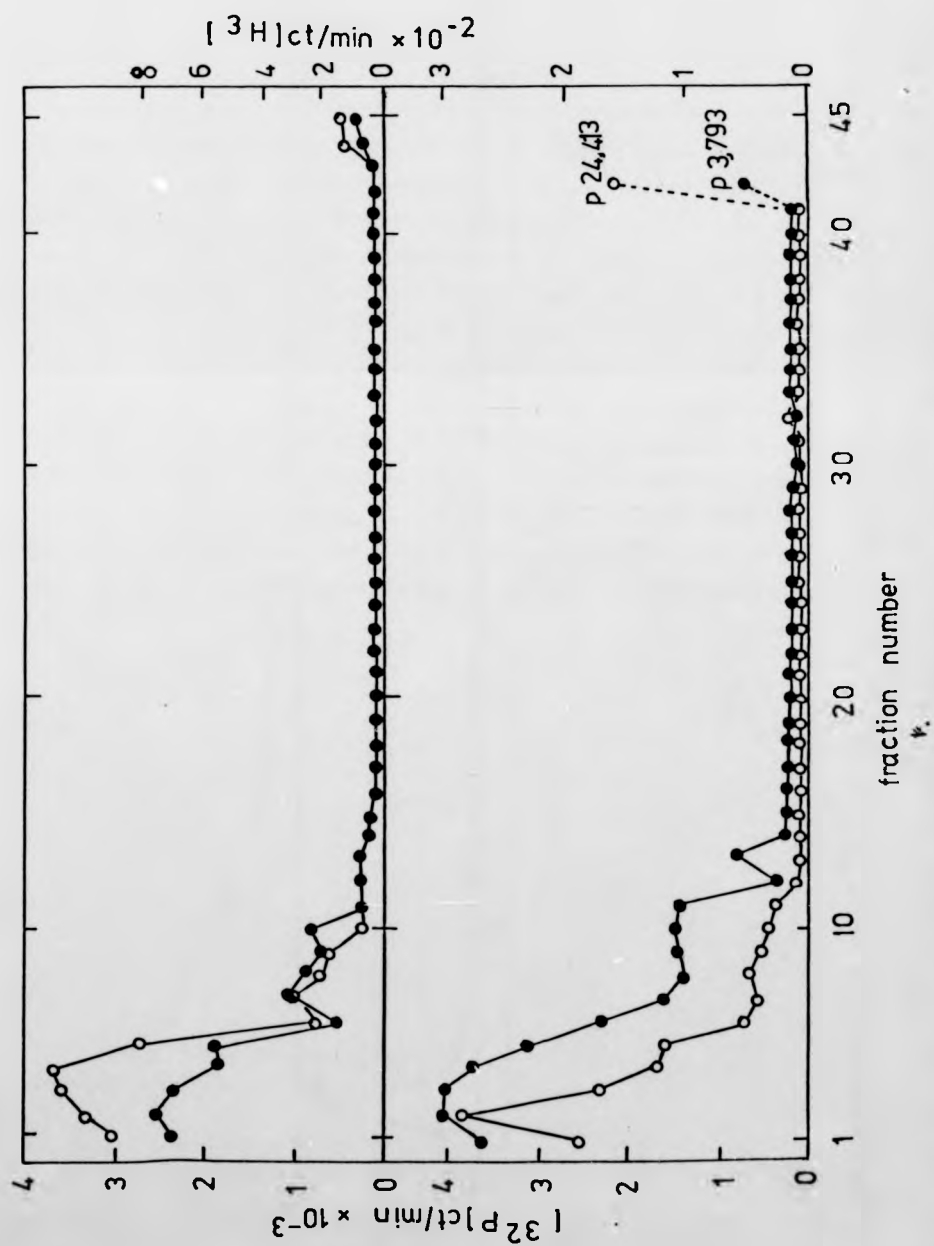


Figure 25. S-value of the replicate on linear sucrose gradients.

The procedure was the same as for Figure 22 except that the material was layered directly on the linear gradients and not incubated in the fraction mixture. The gradients were fractionated by aspiration from the top into 500 $\mu$ l of assay mixture. The fractions were then incubated at 35°C for 35min before TCA insoluble radioactivity was determined. The label was [ $^{32}$ P]-GTP.

Panels a and b : detergent treated.

Panel c : untreated.

In the experiments shown in panels b and c the material analysed came from the same 10 roller bottle cultures. Panel a was a repeat of b to check the S-value.

The arrows are the position of MV 150S marker nucleocapsids, prepared according to Methods, and run in parallel gradients (with or without detergent).



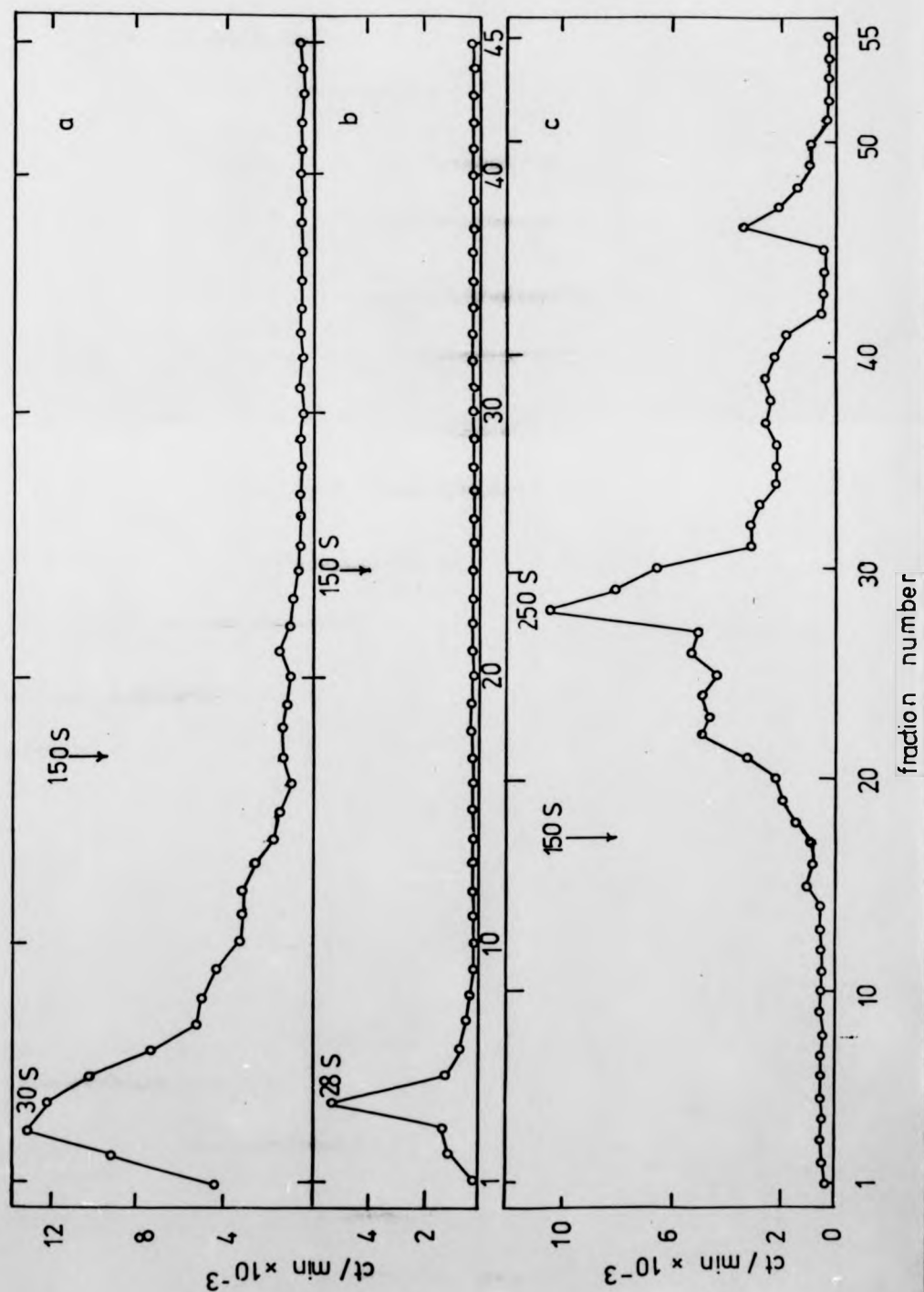
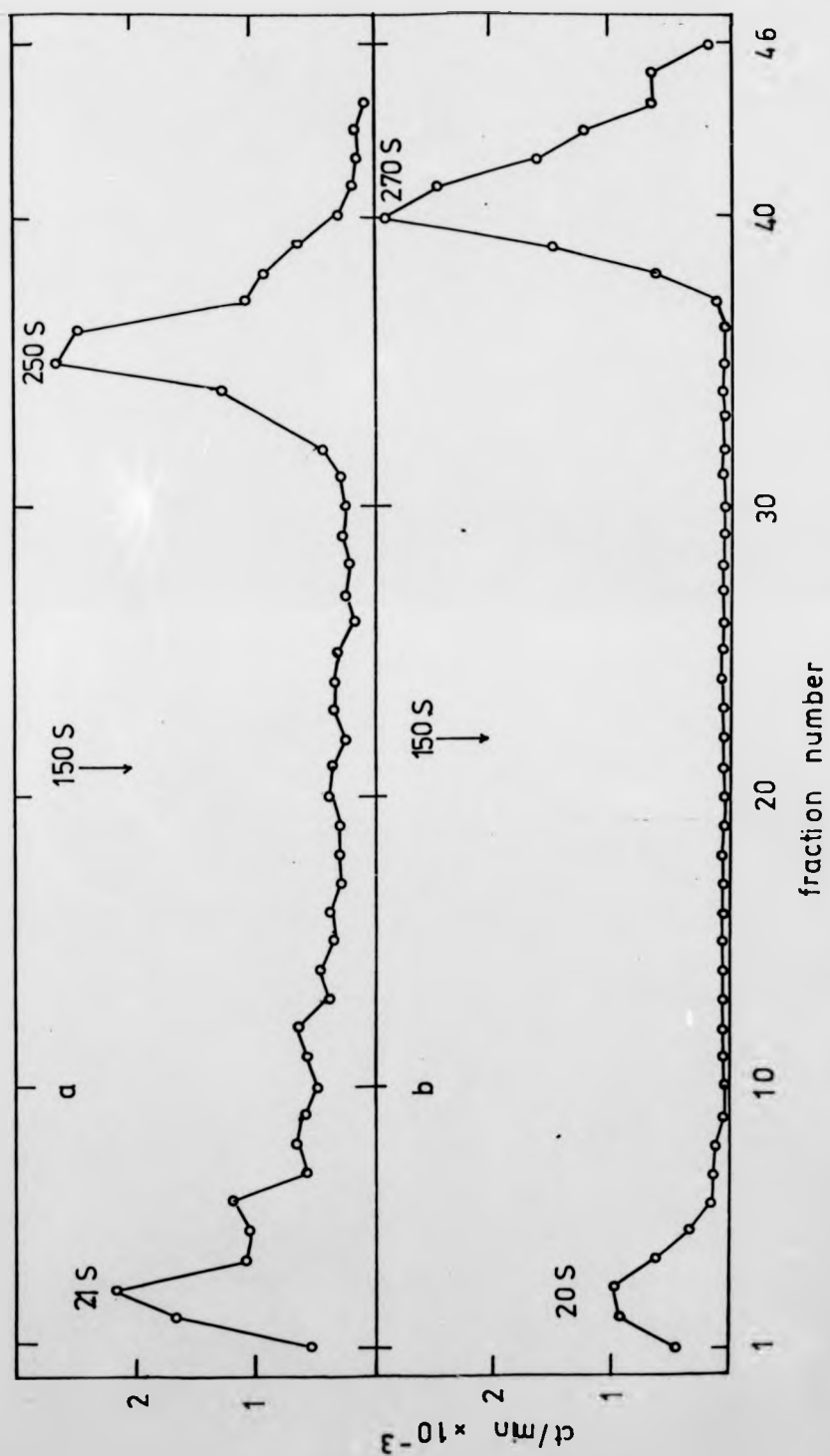


Figure 26. S-value of replicase on linear sucrose gradients. The procedure was as Figure 25 in the presence of Triton N-101. The panels (a and b) show two separate experiments where a visible band was present near the bottom of the tube. The arrow shows the position of SFV 150s marker nucleocapsids run in parallel gradients.



## 9. Attempted binding of replicase to various affinity columns

It was thought that the replicase might bind to specific ligands attached to an inert matrix. The ligands chosen were those which might be expected to be substrates, or to be recognised by the replicase i.e. 2 derivatives of ATP, poly(C), oligo (dT) and oligo (dT) bearing attached 42S RNA. It was determined whether the replicase, at several stages in its purification, would bind to these columns.

### (i) Immobilized ATP columns

As shown in Figure 27, the detergent treated replicase preparation, from an isopycnic gradient, pelleted through a 1ml 5% (w/v) sucrose cushion in RSBM did not bind to ATP linked via its ribosyl group to Sepharose 4B (see Methods). Heavy meromyosin binds to this column and can be specifically eluted with ATP (Lamed, Levin and Oplatka, 1973).

A replicase preparation did not bind to ATP linked through its N<sup>6</sup> position to Bio-Rad Affi-Gel (Figure 28). This column was eluted with both 10mM ATP (to test for specific binding) and 500mM KCl (to test for non-specific ion-exchange effects). Similar N<sup>6</sup>-(6 aminohexyl)-nucleotide ligands have been used to study the selective binding of NAD<sup>+</sup> and NADP<sup>+</sup> dependent dehydrogenases (Hipwell, Harvey and Dean, 1974; Brodelius, Larsson and Mosbach, 1974).

### (ii) Poly(C)-Sepharose columns

Q $\beta$  bacteriophage induces an RNA replicase which can accept synthetic ribocopolymers containing cytidylate residues as a template for the synthesis of a complementary strand (Küppers and Sumper, 1975); EMC virus induced RNA replicase will accept poly(C) as a template (Rosenberg *et al.*, 1972) and the virus RNA contains a poly(C) tract of 85 to 90C (Porter, Carey





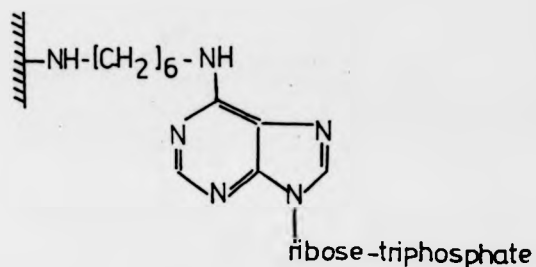
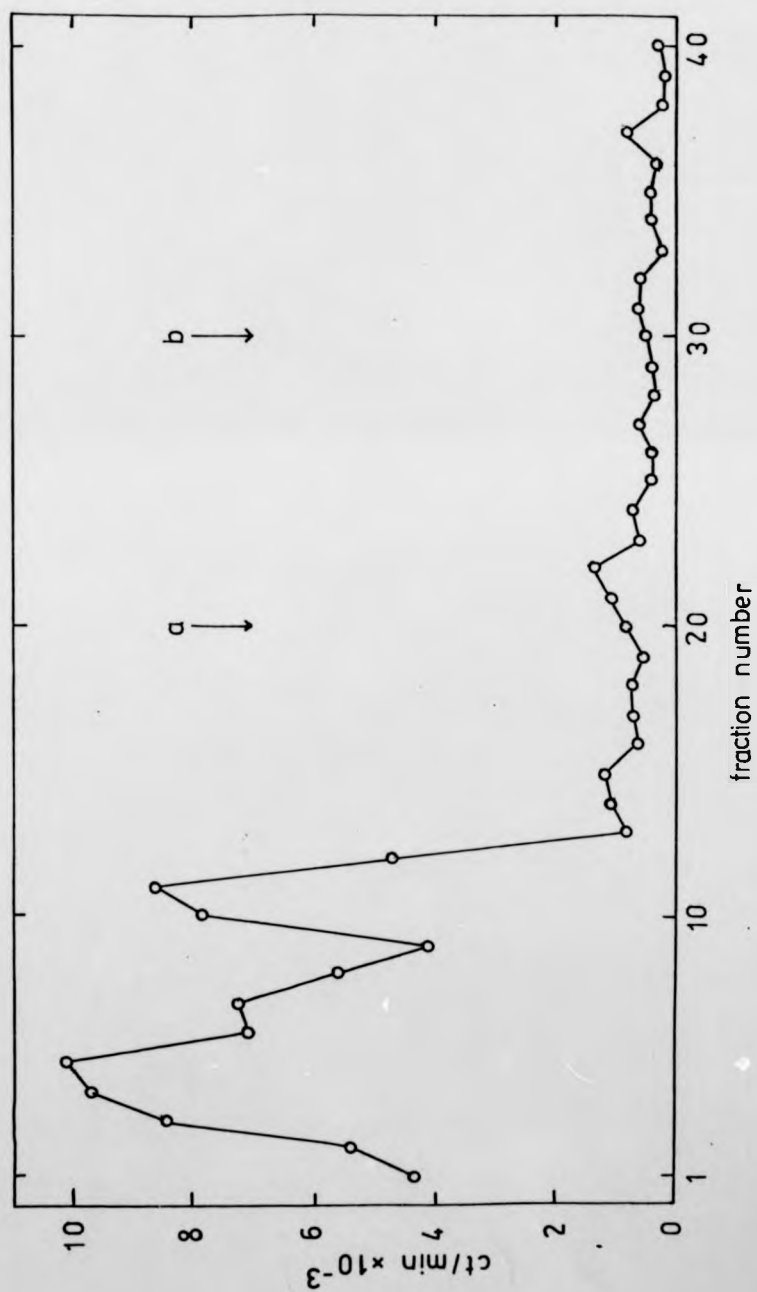


Figure 28. Chromatography of detergent treated replicase enriched band, from an isopycnic gradient, on an immobilized ATP column. The replicase fraction was prepared exactly as described for Figure 27. ATP was coupled, via the amino group of adenine, to Bio-Rad Affi-Gel 10 as described in Methods. The structure of the complex is shown above. The column was pre-washed with 20ml of RSBM, and chromatography was as described for Figure 27 except that the column was washed with 5ml RSBM, 2.5ml RSBM containing 10mM ATP, and finally 2.5ml RSBM containing 500mM KCl.

Arrow a : buffer changed to RSBM containing 10mM ATP.

Arrow b : buffer changed to RSBM containing 500mM KCl.



and Fellner, 1974); Avian Myeloblastosis virus RNA-dependent DNA polymerase binds to poly(rC)-agarose (Marcus, Modak and Cavalieri, 1974), and therefore the binding of SFV replicase to poly(C)-Sephadex was investigated. Although the enzyme will not accept poly(C) as a template (Table 2), since it was template independent, it was considered possible that the complex might bind to poly(C). It was found, however, that no [ $^{35}\text{S}$ ]-methionine labelled protein from infected cells, no viral specific labelled RNA and no replicase activity bound to the column (Figure 29). That the 25S replicase complex from a 5-65% (w/v) sucrose gradient does not bind to poly(C)-Sephadex does not allow the conclusion that a template independent replicase would not accept poly(C) as a template. The divalent cation requirement for such a template may be different (e.g. the template specificity of Q $\beta$  replicase is lost in the presence of  $\text{Mn}^{2+}$  (Stavis and August, 1970)).

(iii) Oligo(dT)-cellulose columns

30% of the RI molecules in infected cells contain poly(A) (Bruton and Kennedy, 1975), and if this proportion of RIs in the replication complex have poly(A) tracts, then it should be possible to bind the replicase bound to the RI to an oligo(dT) column and specifically elute it. The replicase enriched band from an isopycnic gradient was treated with detergent and recovered through a sucrose cushion (as described for Figure 27). Chromatography of this material on oligo(dT)-cellulose (Figure 30) did not give any binding of the replicase to the column, it eluted directly off. Table 7 shows that the buffers and temperatures used during oligo(dT)-cellulose chromatography do not cause any significant destruction of replicase activity. There is no variation in the amount of

Figure 29. Chromatography of the 25S replicase complex on poly(C)-Sephadex. Sixteen confluent roller bottle cultures of BHK cells were incubated with maintenance medium containing 1 $\mu$ g/ml AMD for 3hr before infection with SFV. After 1hr adsorption 4 were washed with EDA medium containing 500 $\mu$ Ci [ $^{35}$ S]-methionine per bottle. On harvesting they were combined with 4 unlabelled cultures, infected for the same time. Another 4 cultures were each labelled with 500 $\mu$ Ci [ $^3$ H]-uridine from 3 $\frac{1}{2}$  to 4hr P.I. as described in Methods. On harvesting they were combined with 4 unlabelled cultures, infected for the same time. These two pools of cells were separately fractionated to give the detergent treated replicase complex (25S) on linear sucrose gradients (see Figures 22 and 25a, b). The gradients were unloaded to give 19 fractions of 700 $\mu$ l each. 100 $\mu$ l aliquots were assayed for replicase activity using [ $\alpha$ - $^{32}$ P]-GTP as the labelled substrate. Fraction 3 (from the top) of both gradients had: replicase activity ([ $^{35}$ S]-labelled sample: 30,194 ct/min [ $^{32}$ P]-incorporated, [ $^3$ H]-labelled sample: 19,747 ct/min [ $^{32}$ P]-incorporated).

Panel a. Chromatography of the [ $^{35}$ S]-labelled sample: 300 $\mu$ l fractions were collected, 100 $\mu$ l aliquots of which were assayed for replicase activity, o—o [ $^{32}$ P]-ct/min, ●—● [ $^{35}$ S]-ct/min.

Panel b. 200 $\mu$ l of the [ $^3$ H]-labelled sample was incubated with 500 $\mu$ l assay mixture using [ $\alpha$ - $^{32}$ P]-GTP, and the whole (700 $\mu$ l) chromatographed. 300 $\mu$ l fractions were collected, 100 $\mu$ l aliquots of which were assayed for replicase activity, o—o [ $^{32}$ P]-ct/min, ●—● [ $^{35}$ S]-ct/min.

Panel c. Chromatography of the remaining 300 $\mu$ l of the [ $^3$ H]-labelled sample. 300 $\mu$ l fractions were collected, 100 $\mu$ l aliquots of which were assayed for replicase activity, o—o [ $^{32}$ P]-ct/min, ●—● [ $^{35}$ S]-ct/min.

The poly(C)-Sephadex was prepared as described in Methods. After recovery from glycerol it was washed in the column with 20ml RSBM. The columns were run following the conditions described in Methods for oligo(dT)-cellulose chromatography. The arrows indicate the fraction at which the buffer was changed from 5ml binding buffer to 5ml elution buffer.

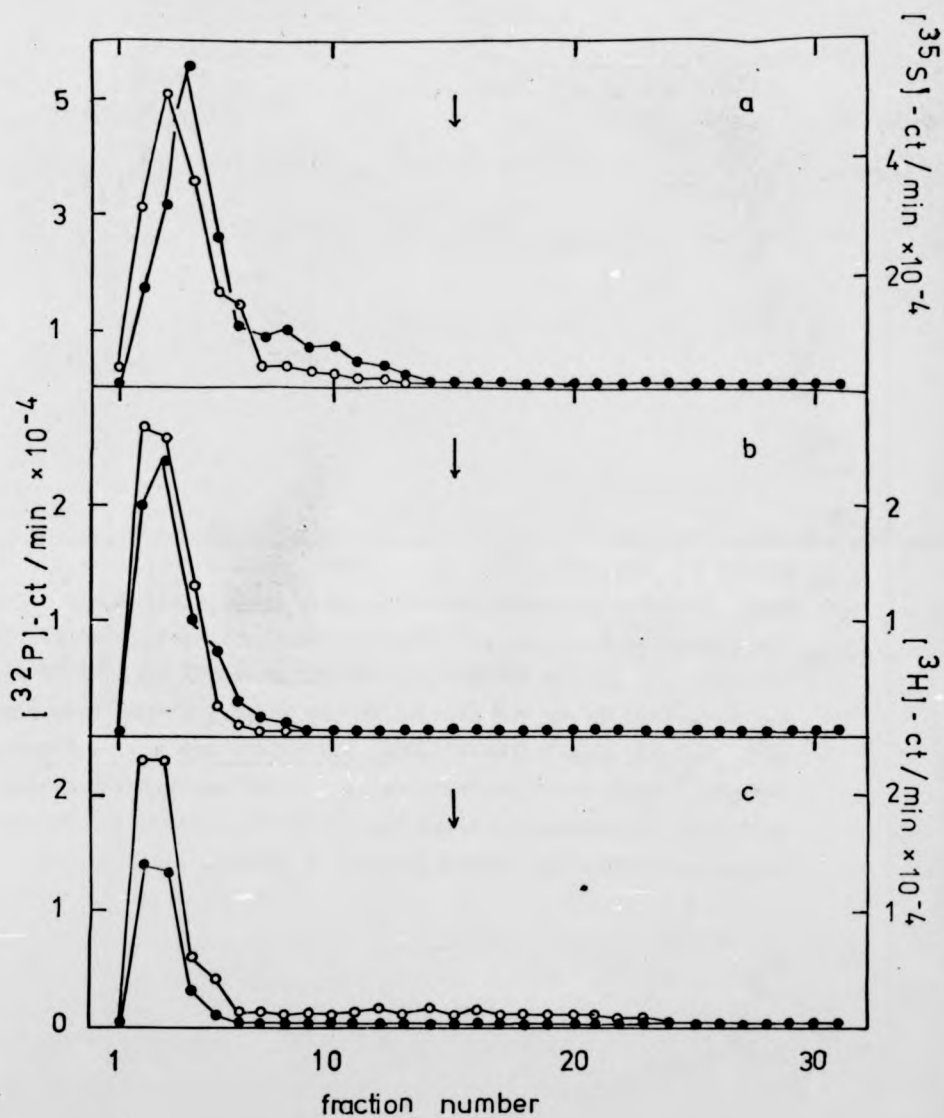
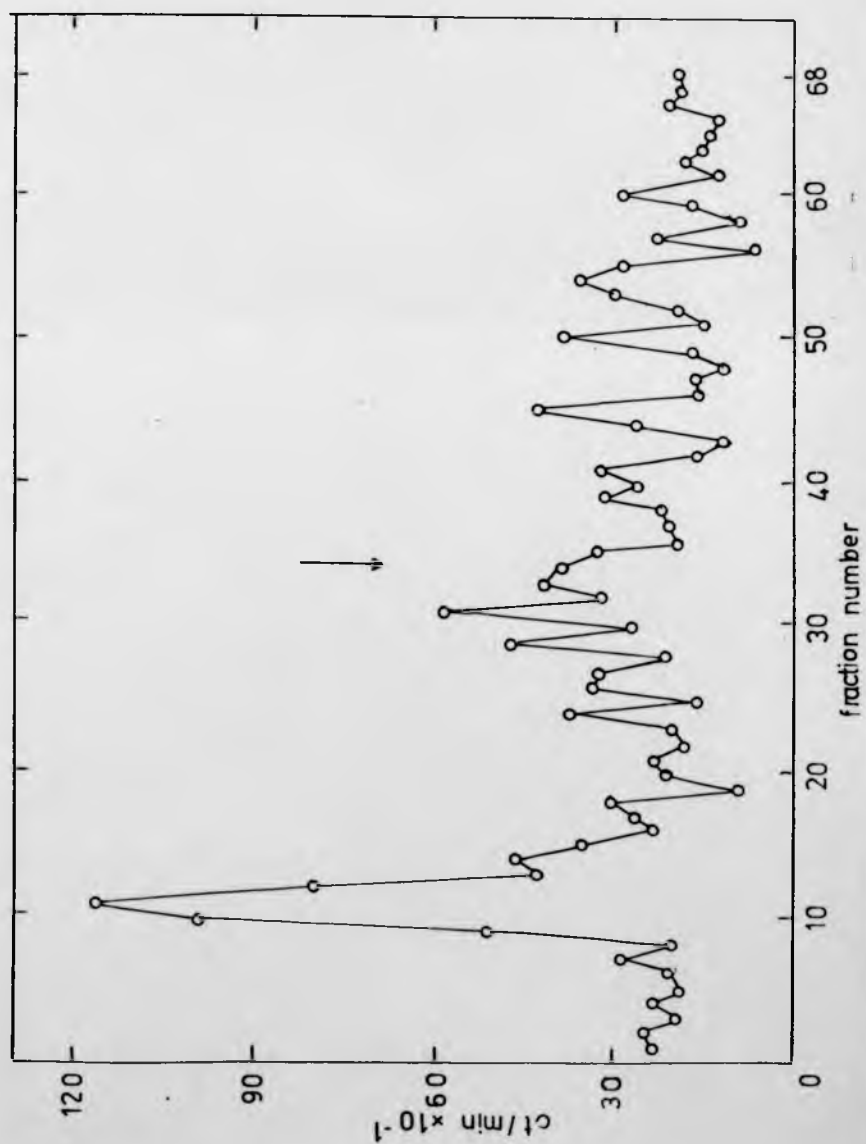


Figure 30. Chromatography of detergent treated replicase enriched band, from an isopycnic gradient, on oligo(dT)-cellulose. The replicase fraction was prepared exactly as described for Figure 27. The oligo(dT)-cellulose was allowed to swell at 30° C in elution buffer before being transferred to the column. It was then washed with 20ml binding buffer at 30° C. The column was run as described in Methods. 250µl fractions were collected and assayed for replicase activity. The arrow indicates the fraction at which the buffer was changed from binding buffer to elution buffer.



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	Incubation temperature		
	0° C	20° C	30° C
RSEM	2,009	2,301	2,476
RSEM + TN101	3,233	1,106	2,059
Binding buffer	1,902	1,398	1,433
Binding buffer without TN101	2,172	1,451	1,778
Elution buffer	1,721	1,279	1,245
Elution buffer without TN101	1,562	1,002	1,697

Table 7. Effect of buffers used in oligo(dT)-cellulose chromatography on replicase activity. The replicase enriched band from an isopycnic gradient, prepared from 12 roller bottle cultures of infected BHK cells, was divided into 3 equal volumes and pelleted by centrifugation. 1 pellet was resuspended in 600 $\mu$ l RSEM, 1 in 600 $\mu$ l binding buffer without Triton N-101 (TN101) and the third in 600 $\mu$ l elution buffer without TN101. These three samples were each divided into 300 $\mu$ l volumes and to one of each TN101 added to 0.1% (v/v). The six samples were each divided into 100 $\mu$ l volumes and incubated at either 0° C, 20° C or 30° C for 15min at which time 250 $\mu$ l of assay mixture was added, and incubation was then at 35° C for 30min to determine replicase activity. Results are expressed as ct/min [  $^3$ H ]-GTP incorporated.

label incorporated other than that expected due to loss of activity during the preincubation.

Synthesis of viral RNA occurs on negative strand templates. The replication complex has been shown, by the RNase experiments described earlier, to contain singlestranded RNA. A proportion of these single-strands must be of negative polarity, and the only negative strand that can be extracted from the cell is of 42S size (Bruton and Kennedy, 1975). Support for these ideas comes from the observations of Thach et al (1974), that the RI of EMC virus contains little double-stranded RNA. Therefore the exposed negative strands of the replication complex should bind to 42S virion RNA. Additionally, the replicase itself must recognise and bind to 42S RNA, and for these reasons the binding of the 25S replication complex to 42S RNA-oligo(dT)-cellulose was investigated. The replicase polypeptide(s) or any exposed negative strands may bind to 42S RNA. As Figure 31a shows 42% of the replicase activity recovered from such a column bound to 42S RNA. If the replication complex from the isopycnic gradient was incubated in the assay mixture before applying to the linear gradient and then the column, only 9% of the ct/min in the TCA insoluble RNA bound (Figure 31b). This may be due to the fact that the pre-incubated enzyme has produced a completely double-stranded product and so contains no exposed negative strands able to bind to positive 42S. Alternatively, at the end of the 30min enzyme assay the replicase polypeptide(s) may not be accessible to more template. Or, the 42S RNA eluted off the column with the replicase (not preincubated) may act as template so giving an apparent binding of 42%.

Figure 31. Chromatography of the 25S replicase complex on 42S RNA-oligo(dT)-cellulose. 4 roller bottle cultures of BHK cells were infected and labelled with [ $^{35}\text{S}$ ]-methionine as described for Figure 29. These cells were combined with 4 unlabelled cultures, infected for the same time, and P15 from them fractionated on an isopycnic gradient. The replicase active band was pelleted and resuspended in 500 $\mu\text{l}$  RSEM containing 1% (v/v) TN101. 400 $\mu\text{l}$  was analysed on a linear sucrose gradient (see Figure 25). The gradient was fractionated into 10 samples of about 1.3ml, and 100 $\mu\text{l}$  of each assayed for replicase activity. Only fractions 2 & 3 from the top contained enzyme (7,009 & 4,589 [ $^{32}\text{P}$ ]-ct/min respectively). These two fractions were pooled, made 100mM NaCl and then chromatographed. 500 $\mu\text{l}$  samples were collected, 100 $\mu\text{l}$  aliquots of which were assayed for replicase activity.

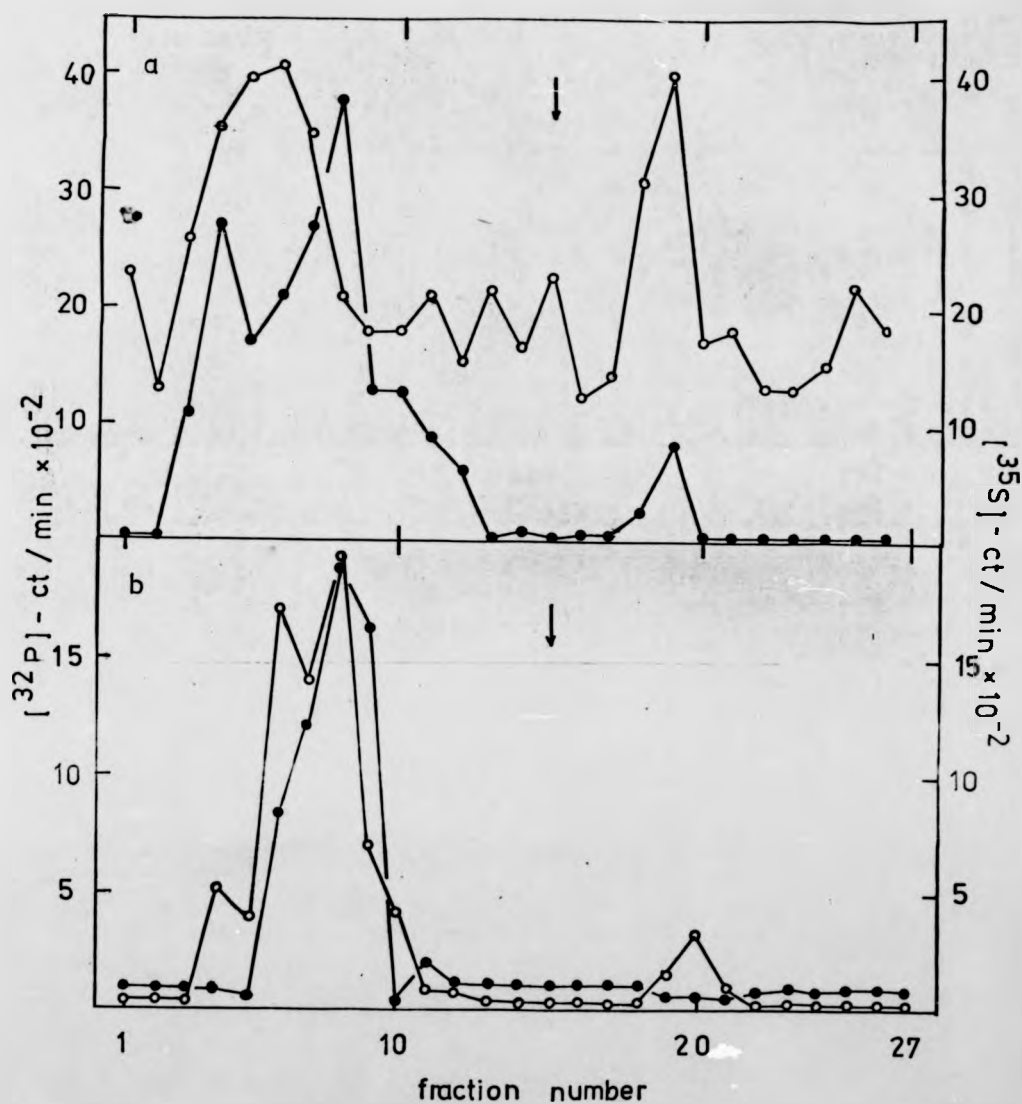
Panel a: ○ — ○ [ $^{32}\text{P}$ ]-ct/min, ● — ● [ $^{35}\text{S}$ ]-ct/min.

The remaining 100 $\mu\text{l}$  of the replicase band was incubated with assay mixture, analysed on a linear sucrose gradient (see Figure 22), and the TCA insoluble ct/min in 100 $\mu\text{l}$  aliquots of 1.3ml fractions determined. Only the top two fractions contained TCA insoluble radioactivity (4,599 & 1,853 ct/min respectively). These two fractions were pooled, made 100mM NaCl and then chromatographed. 500 $\mu\text{l}$  samples were collected, the TCA insoluble radioactivity in 100 $\mu\text{l}$  aliquots was determined.

Panel b: ○ — ○ [ $^{32}\text{P}$ ]-ct/min, ● — ● [ $^{35}\text{S}$ ]-ct/min.

Each oligo(dT)-cellulose column was set up as described in Methods and approximately 900,000 ct/min of [ $^3\text{H}$ ]-SFV 42S RNA (100 $\mu\text{g}$ ) run in at 0°C. Each column was then washed with 2ml binding buffer and the ct/min in the excluded buffer monitored to determine the % binding of the 42S RNA. As shown in Figure 5 this was 60 to 70%. The sample was then run in at 0°C, the column washed with 5ml binding buffer at 20°C, then with 5ml elution buffer at 30°C. The arrows indicate the fraction at which the temperature was raised to 30°C and the elution buffer run in.

40  
30  
20  
10  
15  
10  
5  
[ $^{32}\text{P}$ ]-ct/min  $\times 10^{-2}$



#### 10. Polypeptides associated with RNA replicase activity

The polypeptides, labelled with [ $^{35}\text{S}$ ]-methionine from 0 to 3hr P.I., of various subcellular fractions during the purification procedure were analysed on polyacrylamide gels. Equivalent fractions from mock-infected cells were analysed identically. Figure 32a shows that during the purification procedure there was a loss of polypeptides but that the material loaded onto the oligo(dT)-42S column (lane h) still contained many polypeptides which were not associated with replicase activity. Only 3 polypeptides specifically bound to the column (lane j) and one of these was present in the equivalent mock-infected extract (lane i).

The two larger polypeptides were also visible in the replicase fractions from the isopycnic gradient (lane f) and the linear gradient (lane h) but were absent from the equivalent mock-infected fractions (lanes e and g respectively).

• These polypeptides comigrate with two non-structural polypeptides detected by Dr. C. Clegg (in preparation). Figure 32b (lane a) shows the polypeptides extracted from infected cells labelled from 3 to 3½hr P.I. and then chased for 1hr with cold methionine. Two polypeptides of MWs 90,000 and 63,000 (p90 and p63) were seen to comigrate with the polypeptides which bound to the affinity column (lane b). No structural proteins ( $E_1$ ,  $E_2$  or core) were visible in the replicase preparation. The polypeptide bands in lane b are not as sharp as those in lane a probably because during the purification procedure some proteolysis occurred.

It is pertinent to discuss the as yet unpublished observations of Clegg which have led him to identify non-

structural polypeptides and to postulate a cleavage scheme giving rise to p90 and p63. If infected cells were labelled in 30min pulses starting at zero time P.I. (after 1hr virus adsorption at 4°C) then at 2hr P.I. polypeptides characteristic of infected cells were seen (MW range 100,000 to 200,000 daltons). These polypeptides were seen in both chick and hamster cells. During pulse and chase experiments they disappeared and 2 lower MW polypeptides were seen. Their molecular weights were determined as 90,000 (p90) and 63,000 (p63). Tryptic peptide analysis showed that these polypeptides are distinct from the virus structural proteins and also from host polypeptides isolated from the corresponding position on mock-infected gels. Using an inhibitor of proteolysis (tosyl-phenylalanyl chloromethione), an inhibitor of cleavage (zinc) and an amino acid analogue (canavanine) it was found that the high MW species were not cleaved. These experiments have led Clegg to propose that 42S RNA is translated to give a 200,000 dalton polypeptide which is processed via a 184,000 dalton precursor to give a 150,000 dalton polypeptide which is then cleaved to give p90 and p63. Salt shock experiments (see Clegg, 1975) allowed p63 to be assigned as N-terminal and showed that there is only a single initiation site on the 42S RNA used to make these polypeptides. Tryptic peptide analysis has confirmed that the 150,000 polypeptide is the immediate precursor of p90 and p63. The failure of cleavage in the presence of zinc is correlated with an inhibition in RNA synthesis strongly suggesting that the lower MW polypeptides are involved in RNA synthesis. That these polypeptides comigrate with those purified as replicase polypeptides is good evidence of their function.

Figure 32. Polypeptides associated with subcellular fractions and with purified replicase.

Panel a. Sixteen roller bottle cultures of BHK cells were incubated overnight with Eagle's GMEM containing 1/10th of standard methionine concentration and only 2% (v/v) dialysed calf serum. 3hr before addition of virus the fluids were replaced with GMEM lacking methionine and containing 2% calf serum. AMD was present at 1 $\mu$ g/ml. 8 cultures were infected and 8 mock-infected with SFV in EDA at a m.o.i. of 50 p.f.u./cell. After 1hr adsorption at 4°C the fluids were replaced with EDA containing 750 $\mu$ Ci [ <sup>35</sup>S ]-methionine per roller bottle. The cells were harvested 3 $\frac{1}{2}$ hr later and fractionated as described in Methods. The recovered replicase band and equivalent mock-infected band from the isopycnic gradient were separately resuspended in RSEM containing 1% Triton N-101. Analysis of these bands was by centrifugation at 200,000g for 3 $\frac{1}{2}$ hr at 4°C through 14ml linear 5 to 40% (w/v) sucrose gradients prepared in RSEM containing 1% Triton N-101. The peak fractions having replicase activity were chromatographed through an oligo(dT)-cellulose column exactly as described in Figure 31. Samples taken during this purification procedure were prepared and analysed on slab gels as described in Methods. The dried gels were exposed on Kodirex X-ray film for 4 days.

Lane a. Mock-infected post-nuclear supernatant, 380,000 ct/min.

Lane b. Infected post-nuclear supernatant, 240,000 ct/min.

Lane c. Mock-infected P15, 240,000 ct/min.

Lane d. Infected P15, 250,000 ct/min.

Lane e. Mock-infected "replicase" band from isopycnic gradient, 350,000 ct/min.

Lane f. Infected replicase band from isopycnic gradient, 200,000 ct/min.

Lane g. Mock-infected fraction from linear gradient, 130,000 ct/min.

Lane h. Infected replicase fraction from linear gradient, 170,000 ct/min.

Lane i. Mock-infected bound to column, 63,000 ct/min.

Lane j. Infected replicase bound to column, 120,000 ct/min.

Panel b. BHK cells grown in a unicam vial were infected with SFV in the presence of 1 $\mu$ g/ml AMD. At 3hr P.I. the culture was labelled with 100 $\mu$ Ci [ <sup>35</sup>S ]-methionine for 30min and then chased with cold methionine for 1hr.

Lane a. As above. p90, p68 & p63 are non structural virus polypeptides. ( MWs 90,000, 68,000 & 63,000 respectively ). E<sub>1</sub> & E<sub>2</sub> - envelope proteins of the virus, C - core protein of the virus. Sample kindly given by Dr. C. Clegg.

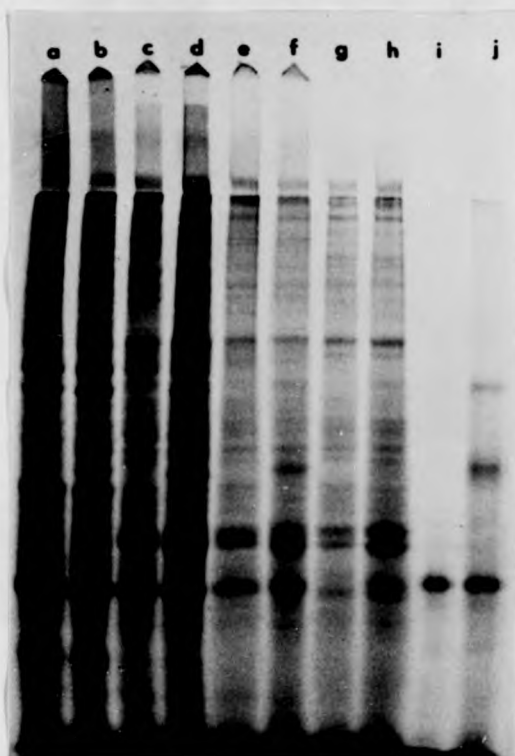
Lane b. Purified replicase equivalent to lane j panel a.

Lane c. Host protein equivalent to lane i panel a.

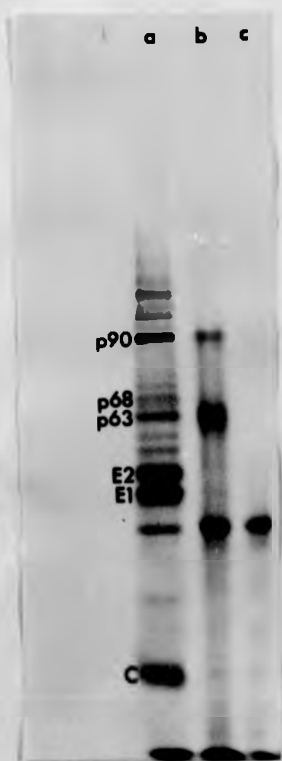
panel a

pa

panel a



panel b





## APPENDIX TO RESULTS

During the course of this research other methods of purifying the replicase were attempted. For clarity of presentation of the most satisfactory procedure, and for fear of including too many "negative" results, these are written as an appendix to the main results section.

### 1. Fractionation of P15 in the presence of Triton N-101

Ten roller bottle cultures of BHK cells were infected and harvested at 4hr P.I. as described in Methods. The pelleted cells were allowed to swell in the presence of 40% (w/v) sucrose in RSBM for 15min at 4°C. P15 was prepared and resuspended in 5ml of 30% (w/w) sucrose in RSBM containing 1% (v/v) Triton N-101. This was fractionated on the standard isopycnic gradient (the other layers were without detergent) and on harvesting there were 2 main visible bands, at 60% (w/w) sucrose and 19% (w/w) sucrose. In addition there was a minor band at 37% (w/w) sucrose. These bands were recovered by centrifugation and each resuspended in 500 $\mu$ l of RSBM containing 1% (v/v) Triton N-101. 100 $\mu$ l aliquots of each were removed and diluted to 300 $\mu$ l with RSBM and 100 $\mu$ l aliquots assayed for RNA replicase activity:

<u>Fraction</u>	<u>Mean ct/min incorporated</u>
"60%" band	23,425
"37%" band	4,313
"19%" band	922
Background	376

Thus the bulk of the replicase activity was at 60% sucrose and not 35% as was found in the absence of detergent.

The "60%" band was layered onto a 6ml 15 to 50% (w/v) linear sucrose gradient prepared in RSBM containing 1% (v/v) Triton N-101 and analysis was by centrifugation at 80,000g for 75min. 200 $\mu$ l fractions were collected into 500 $\mu$ l of assay mixture and then incubated at 35°C for 30 min to determine TCA insoluble radioactivity incorporated. There was a pellet which was resuspended in 200 $\mu$ l RSBM and similarly assayed. The only TCA insoluble radioactivity that was detected was in the pellet: 1,588 ct/min. This represents a loss of replicase activity of 98%. To reduce this unacceptable loss of enzyme activity the linear gradient conditions were varied, otherwise the protocol was identical. As Table 8 shows the best recovery of enzyme activity (16%) occurred when the "60%" band was resuspended in RSBM without detergent and analysed on a gradient lacking detergent. The enzyme obviously becomes very unstable when exposed to detergent for long periods of time, and the little activity recovered represents residual replicase associated with other material and not completely membrane free. The absence of any 25S structure as described in Figures 24 to 26 supports this conclusion.

## 2. Binding of replicase to nitro-cellulose filters

It was envisaged that ribonuclear protein complexes might bind to nitrocellulose filters (Sartorius nitro-cellulose membrane filters, 0.45 $\mu$ m pore size; 25mm dia. V.A. Howe & Co. Ltd., London) so the use of these filters in the place of Whatman GF/C filters was investigated.

P15 containing 100 $\mu$ g of protein was assayed in the standard manner and TCA insoluble counts determined on nitro-

<u>Gradient</u>	<u>1% TN101 in sample</u>	<u>1% TN101 in gradient</u>	<u>ct/min</u>		<u>% recovery</u>
			<u>Gradient</u>	<u>Pellet</u>	
15-50%	+	-	NS	2,911	4
30-65%	+	+	NS	8,143	11
30-65%	+	-	NS	NS	NS
30-65%	-	-	NS	11,846	16

Table 8. Analysis of detergent treated replicase on linear gradients. The "60%" band was prepared from 20 roller bottles containing confluent BHK cells infected with SFV at a m.o.i. of 50 p.f.u./cell as described in the text. This band was recovered and resuspended in 500 $\mu$ l of RSBM, 100 $\mu$ l removed and diluted to 300 $\mu$ l. 100 $\mu$ l aliquots were assayed for replicase activity: mean ct/min 24,736. The remaining 400 $\mu$ l were analysed as shown above by centrifugation at 80,000g for 75min on 6ml linear sucrose gradients. The detergent conditions were varied as shown. The gradients and their pellets were assayed for replicase activity. Results are expressed as ct/min [ $^3$ H]-GTP incorporated and as a percentage of the expected recovery. NS = no significant incorporation above background.

cellulose filters. There was found to be a non-specific binding of [ $^3\text{H}$ ] - GTP to these filters of about 10,000 ct/min (incorporation in presence of enzyme: 20,302 ct/min; incorporation in absence of enzyme: 9,865 ct/min). This non-specific incorporation could be reduced by trimming away the edges of the filter before adding to scintillation fluid: incorporation in the presence of enzyme: 16,123 ct/min; incorporation in absence of enzyme: 3,601 ct/min.

Though the background incorporation was still high it was acceptable provided there was sufficient replicase activity. The binding of the enzyme-product complex to the filter during the assay was next investigated. The filters just fitted inside caps from 2½l bottles, so these caps (after autoclaving) were immobilised on a test tube rack which was incubated in a water bath at 35°C. The filters were placed in caps and the assay mixture containing enzyme added (total volume 350µl) for incubation at 35°C for 30min. After this time the filters were installed in the Millipore filtration apparatus and TCA insoluble counts determined: 6,123 ct/min incorporation in the presence of enzyme: 2,687 ct/min incorporation in the absence of enzyme. There was some apparent binding but this was not very high (about 12% of the incorporation of the same sample assayed in the standard manner and washed through GF/C filters).

Given the information from the previous experiments the binding of P15 to nitrocellulose filters was investigated. P15 in RSBM was passed dropwise through the filters, the filters washed with RSBM and the filters then transferred to caps, overlaid with 500µl of assay cocktail and incubated at 35°C for 30min. The TCA insoluble radioactivity on the

filters was then determined. The following modifications were also tried:

1. Different amounts of P15 from 10 $\mu$ g to 1mg of protein.
2. Increasing the salt concentration in RSBM to 100mM and 500mM.
3. Including 1% (v/v) Triton N-101 in the RSBM.

It was found that unless Triton N-101 was included in the RSBM it was very difficult to filter the material when the amount of protein was greater than 100 $\mu$ g. However, when the detergent was present there was never any recoverable replicase activity. In the presence of RSBM containing 100mM NaCl, and using small amounts of P15 (approximately 50 $\mu$ g) there was some incorporation: 2,909 ct/min above background. This was, however, not reproducible and the method was abandoned. The following observations were made about this system.

(i) There was high non-specific binding of [ $^3$ H] -GTP to the filters. This could be reduced by trimming the edges of the filters after TCA precipitation. The inclusion of 100 $\mu$ M GTP in the TCA did not reduce the non-specific incorporation significantly.

(ii) The filtration rate was highly variable, even between duplicate samples. Detergent conditions which eliminate this and still allow binding need to be determined.

### 3. Lithium chloride solubilization of RNA replicase

Using a polio-virus infected cell extract that had been solubilized with an anionic-nonionic detergent mix, Lundquist, Ehrenfeld and Maizel (1974) were able to pellet replicase activity with 2M LiCl and then isolate a 25S replicase peak on a sucrose gradient. This 25S peak was found to con-

tain predominantly one virus-specific polypeptide. SFV replicase can be treated with the detergent mixture (Figure 10) but the replicase activity is highly unstable. Therefore the "35%" sucrose band from an isopycnic gradient loaded with P15 from 10 infected roller bottle cultures of BHK cells was recovered by centrifugation and resuspended in 3ml of RSBM containing 1% (v/v) Triton N-101. Replicase activity was pelleted by centrifugation at 150,000g for 1½ hr at 4°C. This pellet was treated with LiCl according to the method of Lindquist, Ehrenfeld and Maizel (1974): it was resuspended in 1.5ml of RSBM and an equal volume of 4M LiCl, 10mM tris, 10mM NaCl (pH 8.0) added. The solution was stored at 4°C for 12hr. The RNA pellet was recovered by centrifugation at 2,500g for 10min and resuspended in 2M LiCl buffer during the day, repelleted and dissolved in 10mM tris, 10mM NaCl (pH 8.0) by heating to 37°C for 1min. Table 9 shows that replicase activity was recoverable by this method. Attempts to analyse this material on gradients were not successful as only low levels of replicase activity were pelleted by the LiCl treatment. Despite this failure to recover a 25S replicase as was performed for poliovirus it appears that the method is likely to prove a most useful way of determining the polypeptide composition of the replicase.

Table 9. Replicase activity recovered by LiCl precipitation.

<u>Treatment</u>	<u>[<sup>3</sup>H]-ct/min incorporated in vitro</u>
1. Recovered "35%" band from isopycnic gradient.	12,576
2. Resuspended in RSEM+1% TN101 & pelleted at 150,000g, 1½hr.	7,114
3. Pellet resuspended in 2M LiCl, 4°C for 12hr, centrifuged at 2,500g, 10min.	Pellet: 6,515, Supernatant: NS
4. Pellet resuspended in 2M LiCl, pelleted, dissolved in 10mM tris, 10mM NaCl (pH8.0) by heating to 37°C for 1min.	4,809
5. Layered onto 5 to 65% (w/v) sucrose gradient in RSEM, 200,000g, 3½hr.	NS

Ten infected monolayers from BHK cells from roller bottle cultures were used to prepare the "35%" band. This was recovered and treated as described above and in the text. Aliquots were removed at various stages and assayed for replicase activity. The ct/min incorporated are corrected for the volume of the sample. NS, no significant radioactivity detectable above background level.

### DISCUSSION

The object of this research was to purify and characterize the SFV specified RNA replicase from infected cells. It was decided that the replicase activity of any preparation, analysed for polypeptide composition, should be directly measurable, and that analysis of the polypeptides associated with the 250S replication complex was not a sufficiently rigorous demonstration of the functional polypeptides of the replicase (c.f. Loesch and Arlinghaus, 1975).

Since it was decided to directly measure replicase activity in highly fractionated cell extracts it was necessary that the enzyme assay be capable of giving reproducible high incorporation of precursor into product. The assay described in Methods was found to fulfil these requirements, and using the assay, the levels of replicase activity in crude extracts of BHK cells were extremely high (e.g. about 300,000 ct/min/mg protein/30min incubation c.f. 20,000 for chick cells in which the virus grows equally well: see Table 1). The properties of the assay are that all four ribonucleoside triphosphates are required, the product is a heteropolymer and, in distinction to cellular DNA-dependent RNA polymerases, the activity is found in the cytoplasm and is insensitive to Actinomycin D. These properties are similar to those of other class IV RNA replicases, e.g. poliovirus (Ehrenfeld, Maizel and Summers, 1970), rhinovirus (Yin and Knight, 1972) and Foot and Mouth disease virus (FMDV) (Arlinghaus and Polatnick, 1969a, b).

In the work described here only a very crude subcellular fraction, constituting the post-nuclear supernatant was



competent to synthesize any RNA other than ribonuclease-resistant RNA. This fraction synthesized viral specific single (26S and 42S RNA) and double-stranded RNA (Figure 13). The replicase in this preparation sedimented at 15,000g (giving P15) with enhanced activity, whilst in the 15,000g supernatant little or no replicase activity could ever be detected. The P15 fraction, however, was only competent to synthesize double-stranded RNA. Failure to detect single-stranded RNA as a discrete product may be either due to failure to release single-stranded RNA or to digestion of nascent single-strands by endogenous RNase. Failure to release single-stranded RNA may be due to damage of the transcriptional machinery during fractionation and resuspension or to release of a labile factor into the 15,000g supernatant. Reconstitution of the supernatant and pellet fractions failed to change the RNA profile obtained from the in vitro assay.

During fractionation of the replication complex there would be increasingly greater accessibility of the product RNA to inadvertently released RNase. Great pains were taken to reduce RNase digestion during fractionation by the use of aseptic procedures at 4°C and, in one set of experiments, by the inclusion of polyvinyl sulphate (a ribonuclease inhibitor) in the buffers. Despite these efforts no convincing demonstration of single-stranded RNA synthesis was obtained. However, the experiments with RNase (Figures 14 and 15) show that single-stranded RNA, which is not detected on gradients or gels, is being synthesized during the assay.

The relationship of the various RNA species to each other, as demonstrated in vitro by Michel and Gomatos (1973) appears

to be the same as the product-precursor relationship of the RNA species in infected cells. However, the precise mode of RNA synthesis and, in particular, the role of the RF which may be an extraction by-product (Weissmann, Feix and Slor, 1968; Thach et al., 1974) can only be elucidated by isolating purified replicase, rendering it template independent and then determining the products synthesized from various added templates. It was the aim of this work to progress as far along the path of purification as possible. In view of the many difficulties in purifying animal viral specified intracellular polymerases this task has not been achieved for any virus despite intensive research. It is likely to be a long and arduous path to pioneer.

The replicase activity described here was independent of added template and did not respond to homopolymers added as exogenous template. As the enzyme and its template bound to oligo (dT) bearing 42S RNA it is likely that the enzyme is tightly attached to its template and only completes previously initiated strands. In the case of the addition of homopolymers, the enzyme is likely to have a much greater affinity for the natural template, recognising a specific nucleotide sequence absent in the added template.

Replicase activity is detectable early in infection (1hr after addition of virus) and rapidly reaches a plateau at  $3\frac{1}{2}$  to 4hr P.I. (Time course, Figure 7). At this time infected cells were routinely harvested and the enzyme is stable as evinced by the finding that cycloheximide had no effect on replicase activity at  $3\frac{1}{2}$ hr P.I. (Figure 8). Therefore sufficient replicase molecules have been synthesized by this time and are probably all intimately associated with RNA.

Replicase activity, in the presence of cycloheximide at  $3\frac{1}{2}$  hr P.I., was directly measured and the finding that it was stable confirms results in which RNA synthesis was measured at similar times (Friedman and Grimley, 1969; Wengler and Wengler, 1975b). On going viral protein synthesis (structural proteins) is inhibited, there are no nucleocapsids or CPV-2 formed (Friedman and Grimley, 1969).

Thirty minutes after the addition of cycloheximide at  $1\frac{3}{4}$  hr P.I. there was no detectable replicase activity. This is probably because of destruction of the replication complex during subcellular fractionation as membrane changes are not complete by this time as shown by the distribution of replicase activity over the isopycnic gradient (Figure 21). Moreover, Friedman and Sreevalsan (1970) found that the entry of input RNA into a membrane replication complex required protein synthesis.

#### The membrane bound replication complex

The further characterization of the replicase activity in P15 was by separation of membrane bands on isopycnic gradients (Figures 16 to 21). Replicase activity was present as a membranous band in 35% (w/v) sucrose at 4hr P.I. and had associated viral RNA as shown by labelling cells with [ $^3\text{H}$ ] - uridine from  $3\frac{1}{2}$  to 4hr P.I. At  $1\frac{3}{4}$  hr P.I. the replicase was not present as a discrete band but was spread over the gradient and was associated with input virion RNA. At this time RNA strands of opposite polarity to the virion RNA (negative strands) are being synthesized on template 42S virion RNA. There was no association of replicase activity on the isopycnic gradient with input virion structural proteins.

The pulse labelling experiments show that the bulk of the

RNA is made at the site of the RNA replicase activity and, over 30 min, is not exported elsewhere. There is associated single-stranded RNA with the membrane band on the isopycnic gradient further supporting the contention that the replicase isolated from it is normally competent to synthesize all viral RNA species (Figure 19 and Friedman et al., 1972).

Thus the SFV specified replication complex, banding at 35% sucrose, has associated template, RNA replicase and newly synthesized RNA. There are no input virion structural proteins associated with this complex which may be related to the cytopathic vacuoles observed in the electron microscope and believed to be the site of viral RNA synthesis (Friedman et al., 1972; Grimley et al., 1972).

Once the replicase band on the isopycnic gradient had been established as the replication complex its S-value was then determined. This was achieved by velocity gradient centrifugation through 5 to 65% linear sucrose gradients. The S-value was found to be approximately 250 and this visible band had associated replicase activity, in vivo labelled RNA and product RNA synthesized in vitro. Detergent treatment converts this to a 25S complex which still has associated template and progeny RNA as well as RNA replicase activity. Presumably the 25S structure is the "unit of RNA production" which is inserted into membranes. This finding demonstrates for the first time in alphavirus infected cells a replication complex which can be solubilized by detergent and thereby reduced in size. Interestingly, the flavivirus Saint Louis Encephalitis induces a membranous structure of 250S in the cytoplasm of infected cells which is the site of RNA synthesis (Qureshi and Trent, 1972). In other class IV viruses there is evidence of membranous replication complexes: FMDV and mengovirus induce a

100-300S and a 20-70S replication complex (Arlinghaus and Polatnick, 1969b; Arlinghaus, Syrewicz and Loesch, 1972); detergent treated cytoplasmic fractions from poliovirus infected cells release similar sized structures (Caliguiri and Mosser, 1971; Caliguiri, 1974; Lundquist, Ehrenfeld and Maizel, 1975).

It seems that a replication complex of about 250S similar to the one described here may be present in many virus infected cells. For SFV this 250S structure, which is probably related to CPV-1, is the site of RNA synthesis. The assembly of nucleocapsids, which occurs later in infection, occurs on a different structure, CPV-2 (Grimley, Berezesky and Friedman, 1968).

#### Polypeptides associated with the replication complex

Three in vivo labelled polypeptides bound to the oligo (dT)-cellulose-42S RNA column and were eluted in the fractions having RNA replicase activity. The MWs of two of these (63,000 and 90,000) are equivalent to 2 polypeptides detected in vivo and translated from the 5' (non-structural) region of the 42S genome RNA (Clegg and Kennedy, in preparation). That an entirely independent study should show 2 non-structural virus polypeptides of the same MW as 2 of the polypeptides associated with RNA replicase activity which bound to the double-affinity column strongly suggests that these are the true replicase polypeptides, and together with the observations of Lundquist, Ehrenfeld and Maizel (1965) on the detergent-solubilized, LiCl precipitated 25S poliovirus replicase activity, provide the best evidence so far obtained for the identity of non-structural polypeptides as replicase components of class IV animal viruses.

Besides the two virus specific polypeptides visible on gels there is also another band which is probably a host polypeptide. The presence of 2 virus specific polypeptides fits well with the 2 complementation groups of virus defective in RNA synthesis (Burge and Pfefferkorn, 1966) and with mutants having different ratios of 26 and 42S RNA synthesis (Atkins, Samuels and Kennedy, 1974). It is interesting that Friedman and Sreevalsan (1970) and Sreevalsan (1970) have speculated that a host protein is involved in the association of viral RNA with membranes. They found that interferon had no effect on the association of viral RNA with membranes, whilst cycloheximide prevented it. This protein may have an affinity for viral RNA and so bind to the oligo(dT)-cellulose-42S RNA column. Interestingly, the Q $\beta$  purified replicase requires a host factor for the synthesis of negative strands but not positive strands (Eoyang and August, 1974).

The polypeptides identified as replicase candidates for SFV, mengo, polio and EMC viruses are shown in Table 10. Also included are the definitively identified subunits of the Q $\beta$  replicase.

#### A model for the replication of SFV RNA

The two polypeptides which we believe to be the replicase polypeptides are probably involved in transcriptional control of RNA synthesis. The existence of such transcriptional control is suggested by mutants which have different ratios of 26S and 42S RNA synthesis (Atkins, Samuels and Kennedy, 1974) and by two complementation groups of mutants defective in RNA synthesis (Burge and Pfefferkorn, 1966). Further, the synthesis of 26S RNA varies during infection (Levin and Friedman, 1971; Martin and Burke, 1974).

Table 10. the MWs of the polypeptides associated with RNA replicase activity of some class IV viruses.

VIRUS							REF
QB		70-74,000 30S protein	62-65,000 viral		47,000 Tu	30-35,000 Ts	Eoyang & August, 1974
EMC		72,000	65,000	57,000	45,000	35,000	Rosenberg <u>et al.</u> 1972
MENGO				56,000 viral	40,000 host		Loesch & Arlinghaus. 1975
POLIO				58,000 viral			Lundquist, Ehrenfeld & Maizel, 1974
SFV	90,000 viral		63,000 viral		40,000 host		

As the messenger for the structural proteins of the virion, it is "amplified". In this respect, SFV differs from poliovirus in which only one polypeptide has been identified as a replicase component and which has no subgenomic RNA.

A replication scheme (Figure 33) has been constructed in which there is envisaged to be a sequence of nucleotides at the 3' end of the 42S positive, genomic strand which is recognised by the replicase. A complementary sequence exists at the 5' end so allowing circularization (Hsu, Kung and Davidson, 1973). Upon transcription by the replicase the 3' end of the strand complementary to the genomic strand (the negative strand) will contain the replicase recognition sequence. There must be an internal sequence at the start of the 26S RNA sequences. This sequence is not recognised by the replicase. Its complement, however, on the negative 42S RNA is recognised by the replicase, providing the internal sequence for the synthesis of 26S RNA. The two recognition sequences have different affinities for the replicase so regulating the amounts of 26S RNA and 42S RNA synthesized (possibly one replicase polypeptide recognises one sequence, one the other). Mutants differing in the amounts of 26S and 42S RNA synthesized (Atkins, Samuels and Kennedy, 1974) would have alterations in one of the two virus polypeptides of the RNA replicase.

The following observations support this model.

1. Two virus specific replicase polypeptides have been identified in this work. Possibly these are the agents of transcriptional regulation. The existence of two different recognition sites could be determined by binding purified replicase to 26S and 42S RNA and sequencing the protected fragments.
2. Circular forms of Sindbis RNA (Hsu, Kung and Davidson, 1973)



Figure 3}. A model for SFV RNA replication.

The replicase recognition sequence at the 3' end of the 42S virion strand is VUTSR. Its complement at the 5' end is vutsr. The negative strand produced by replicase transcription from the virion RNA has the replicase recognition sequence VUTSR at its 3' end. An internal recognition sequence, NMlKJ, exists at the start of the 26S sequences on the negative strand. Its complement on the positive strand (nmlkj) is not recognised by the replicase.

The dotted circles represent the replicase molecules.

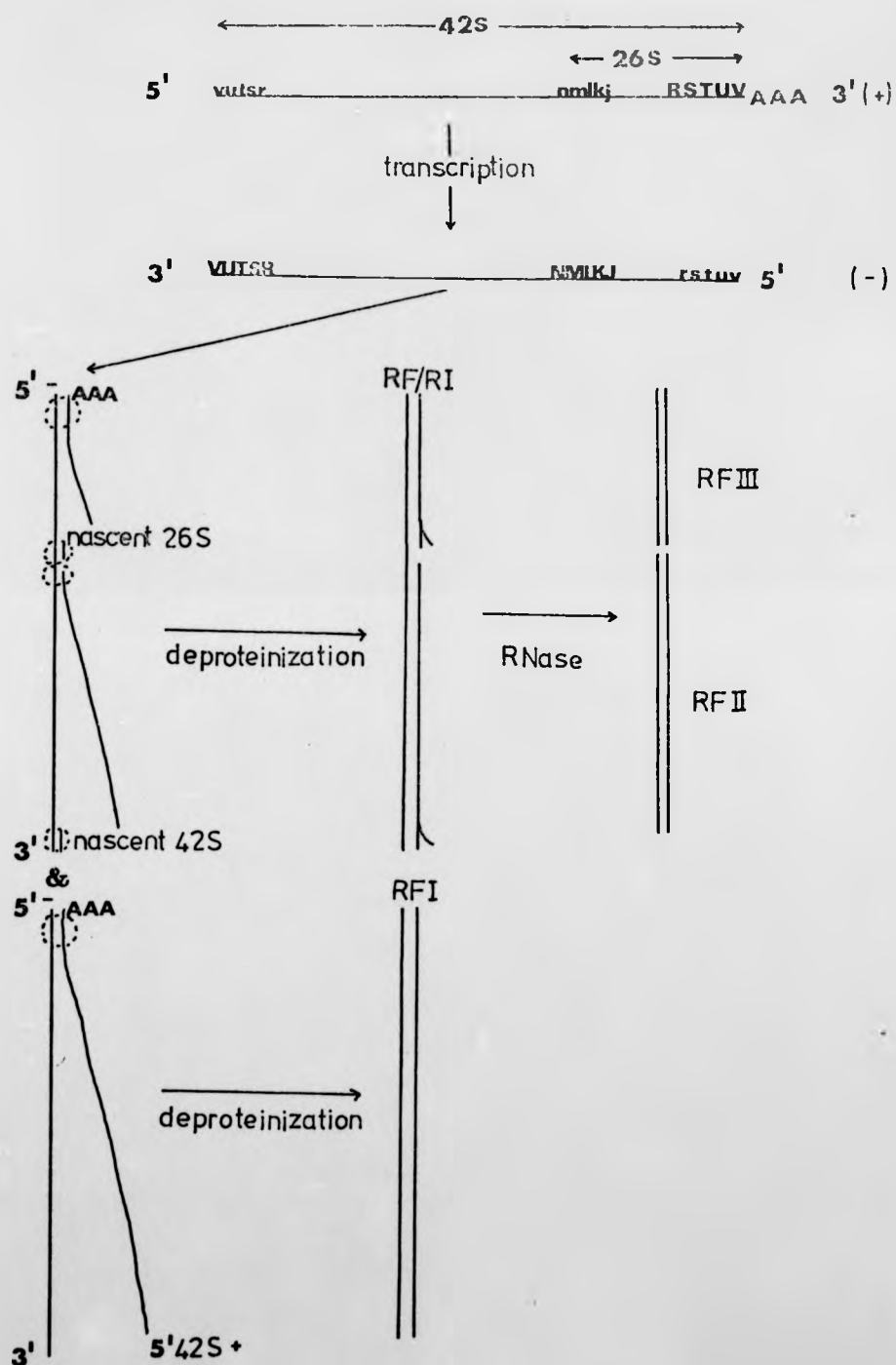


Figure 33 A model for SFV RNA replication

and SFV (S.I.T. Kennedy, personal communication) have been seen.

3. Only negative strand of the 42S size has been isolated from the infected cell (Bruton and Kennedy, 1975). There is no evidence for the existence of RNA of the size required if 26S was derived from cleavage of 42S.

4. The protein bound to the replicase binding site of the 26S RNA would prevent reannealing of the loosely-complexed complementary strands during extraction, and on deproteinization would leave a gap in one strand. This gap would be accessible to RNase thus producing RFII and RFIII in equimolar amounts as observed by Simmons and Strauss (1972a).

5. Cells infected at 42°C with the ts24 mutant of Sindbis (Scheele and Pfefferkorn, 1969b) synthesized very little 26S RNA and the double-stranded cores of the RI consisted of only one major RNA species (Segal and Sreevalsan, 1974). Treatment of normal RI with RNase gives multiple forms of ds RNA cores (Martin and Burke, 1974 ; Segal and Sreevalsan, 1974).

This model may be applicable to other viruses which have subgenomic RNAs, e.g. broome grass mosaic virus (Shin and Kaesberg, 1973) and tobacco mosaic virus (T. Hunt, personal communication).

The model needs to be considered in the context of two stages in the multiplication of SFV: the early stage up to about 3hr P.I. when replicase and negative strands are produced, and the late stage after 3hr P.I. when virus structural proteins are translated from the 26S RNA leaving the 42S RNA free for encapsidation into virus particles. The cycloheximide data is interpreted as showing that the replicase is not modified by newly synthesized proteins after 3hr P.I., and since viral RNAs are produced normally after this time in the presence of

cycloheximide, there must be fully functional replicase present by 3hr P.I.

The oligo(dT)-cellulose-42S RNA column

The binding of RNA containing poly(A) tracts to oligo (dT)-cellulose is well established. The conditions described here enable this to be achieved without SDS. This gives an elegant technique for the study of the binding of proteins and complementary RNA to immobilized RNA. Proteins that might bind to RNA include nucleases, all kinds of polymerases, indeed any protein for which RNA is a template or which has an affinity for RNA. It is likely that these proteins could be eluted in an active form as can SFV replicase. The replicase was purified as much as possible before applying to the column so that no other RNA recognising enzymes could be present. A rapid procedure for the purification of SFV replicase could be devised if the DOC : Triton N-101 solubilized P15 enzyme could be stabilized. This preparation could then be passed down the column and purified replicase prepared in large quantities. Given such a preparation there are many possible experiments. It would be possible to bring the knowledge of animal viral RNA replicases to the level of the Q $\beta$  replicase (see Eoyang and August, 1974).

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